How to Shape Living Structures –
Factors Controlling Spindle Geometry

Grant Application
Strategic Partnership
Princeton University - Humboldt-Universität

submitted by

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PETRY & REBER
ABSTRACT
The goal of this research proposal is to understand the way in which cells engineer larger scale structures, in particular the mitotic spindle. The mitotic spindle’s function is to precisely partition the genetic material into two daughter cells. The geometry of the spindle is inherently linked to its function. While the length of the spindle determines the distance over which chromosomes will be segregated, the bipolar structure of the spindle determines the directionality of chromosome movement. Spindles that are too short can lead to chromosome missegregation. Similarly, multipolar spindles can lead to chromosome missegregation and genomic instability, a hallmark of cancers. While the last decades were instrumental in putting together the parts lists of individual organelles, it is still unknown which principles organize these mesoscale structures. Recent important biological insights from our own work and from that of many others has underscored the power of interdisciplinary studies, combining cell biological methods with biophysical techniques and computational modeling. We feel strongly that the most productive and impactful scientific advances on the fundamental problem of intracellular organization will come from expanding the frontiers of such collaborations, bridging international and disciplinary boundaries. Here, we describe a unique international collaboration that combines biochemical reconstitution, structural biology, biophysical measurements and modeling, and robust analysis of phenotypes at the cellular level. In particular, we wish to understand (1) how the biochemical heterogeneity of tubulin effects spindle organization, how the correct (2) number and (3) length of microtubules is regulated to build a spindle of the correct size. An important objective of our proposal continues to be graduate and postgraduate training including lab exchanges. Taken together, the proposed work will provide important insights into the physical principles that underlie the organization of the mitotic spindle as a molecular machine, which is likely to have important implications for its function in cell proliferation and molecular origins of diseases.

KEYWORDS
Mitotic spindle, mesoscale organization, biophysics, in vitro reconstitution, systems biology
1. OVERVIEW & MOTIVATION

Living cells contain myriad internal substructures that play a wide variety of vital roles for life. A particularly complex organelle is the mitotic spindle, which functions to precisely partition the genetic material into two daughter cells. This makes the spindle an essential structure for cell proliferation and procreation. Errors during chromosome segregation can result in chromosome missegregation, aneuploidy, and cancer. Uncontrolled, cancerous cell growth is typically treated by disrupting spindle organization, making the mitotic spindle a primary therapeutic target for human health. Despite its importance, and being among the most conspicuous cellular structures observed by early light microscopists (Flemming 1882, Wagner R. 1835), there remains a major gap in our understanding of the molecular and biophysical mechanisms by which the mitotic spindle assembles and functions in chromosome segregation. This proposal will tackle this problem by building a scientific network between Princeton University and Humboldt University, leveraging the synergistic expertise of researchers at both institutions to shed light on the fundamental mechanisms underlying spindle assembly and function.

CURRENT LIMITATIONS

The last several decades have been instrumental in advancing our understanding of the function of macromolecular complexes, such as RNA polymerase, DNA polymerase, or the ribosome. However, we still lack any mechanistic understanding of the molecular biophysical principles underlying mesoscale structures such as the mitotic spindle. The next challenge will be to uncover how molecular machines that act at the Angstrom scale are coordinated in space and time to create physiological structures in the \( \mu \text{m} \) scale that enable cell function. This is particularly demanding for membrane-less structures, such as the mitotic spindle, which rely on self-organizing and biophysical principles that remain to be uncovered. These limitations are too large to be tackled by a single laboratory, and new solutions need to be found in order advance human knowledge and health better.

2. INNOVATION & OBJECTIVES

Metaphase spindles are complex structures that can reach 50 \( \mu \text{m} \) in length and can consist of thousands of dynamic microtubules, which constantly exchange subunits and binding proteins with the surrounding cytoplasm. Despite these incessant molecular dynamics, spindles achieve a steady-state size and shape characteristic for each cell type. Decades of work have shown that microtubule dynamics, nucleation, and transport are critical for spindle assembly. These dynamic properties of microtubules are modulated by more than 200 different accessory proteins known as microtubule associated proteins (MAPs) and motors. While the advent of single molecule techniques has recently allowed us to study the biochemical activities of these individual proteins, we still lack an understanding of how the
combined effects of microtubule nucleation, dynamics, and force generation give rise to a spindle. Mesoscale structures, such as the spindle, are generated from thousands of individual molecules in a coordinated fashion. In order to understand how molecules and macromolecular complexes achieve this, it will be critical to first uncover how these factors work on a molecular level. In a second step, we will study the well-characterized factors in complex cellular assemblies. By combining our expertise in structural biology, biochemistry, and biophysics, our team will address the following major outstanding questions:

- How does the biochemical heterogeneity of αβ-tubulin influence spindle morphology?
- How is the correct number and length of microtubules regulated to build a spindle of the correct size?
- What are the biophysical principles that give rise to spindles of defined shape and size?

We will systematically study these questions at three different levels:

**AIM 1. BIOCHEMICAL HETEROGENEITY OF αβ-TUBULIN (including preliminary data)**

The metaphase spindle is a bipolar array of microtubules assembled from dimeric αβ-tubulin subunits. Microtubules have the inherent property to switch between phases of growth and shrinkage, a process termed ‘dynamics instability’. While we know how MAPs and motors can modulate the dynamic instability of microtubules, we do not know if and how the heterogeneity in tubulin biochemistry contributes to modulate microtubule dynamics in health and disease. Cells express different tubulin genes (in humans for example 8 α- and 9 β-tubulins), encoding distinct isotypes. In addition, tubulins carry a number of post-translational modifications (PTMs), such as acetylation, polyglutamylation, phosphorylation, and (de)tyrosination. The combination of different isoforms together with multiple PTMs gives rise to a staggering complexity (Figure 1) of a cells tubulin proteome, which so far has not been studied systematically.

*Figure 1: Sources of Tubulin Heterogeneity. A Different tubulin isoforms are expressed, often in a cell type-specific manner. B Tubulins are modified posttranslationally, including acetylation, polyglutamylation, phosphorylation, and (de)tyrosination. C Microtubules can have a different number of protofilaments, often 13, 14 or 15, resulting in differences in microtubule diameter.*
We here propose to study the influence of tubulin biochemistry on spindle morphology in a system, that allows easy comparison between the effect of tubulin heterogeneity, namely the two related species Xenopus laevis (Xl) and Xenopus tropicalis (Xt). Egg extracts of Xenopus laevis, the African clawed frog, have been instrumental in identifying the biochemistry of spindle assembly but lately also mechanisms of spindle size control and scaling (reviewed in Levy and Heald 2015). Xenopus laevis has a small sister, Xenopus tropicalis, which is smaller in body size, has smaller cells and smaller organelles (Brown 2007). Spindles assembled in Xl extracts are up to 50µm, while spindles assembled in Xt extracts are ~30% shorter. Mixing Xl and Xt extracts revealed a dynamic, dose-dependent regulation of spindle size by cytoplasmic factors (Brown et al., 2007). This type of study was subsequently used to identify a limited number of individual factors involved in size control, such as Katanin (Loughlin 2011) or TPX2 (Helmke 2014). However, a systematic study on how spindle size is controlled is missing. We hypothesize that tubulin heterogeneity is a major factor determining spindle size, because tubulin constitutes the framework and the heart of the spindle. We therefore will study the contribution of the ‘tubulin code’ to size control.

During the last 50 years, almost all biochemical studies have used tubulin purified from mammalian brain tissue with the consequence of studying heterologous system. We have recently designed an affinity matrix, which binds tubulin in an organism-agnostic manner with very high efficiency. The eluted, highly purified tubulin is fully functional as it can efficiently assemble into microtubules as verified by dynamic growth assays and electron microscopy (Widlund 2011).

Figure 2: Tubulin Purification from A X. laevis and B X. tropicalis egg extracts.
During our joint stay at the MBL in Woods Hole in 2016, which harbors the National Xenopus Resource (NXR), we had access to both, eggs of Xenopus laevis and Xenopus tropicalis. We purified the tubulin proteomes of eggs from Xenopus laevis and — for the first time - Xenopus tropicalis (Figure 2). Preliminary data, which we plan to publish in a joint article, include

- Analysis by mass spectrometry revealed that the egg extracts of X. laevis and X. tropicalis have similar but significantly different tubulin isoforms (see Table below).

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- Western blot analysis revealed that there is no difference in the PTMs (Figure 3).

**Figure 3**: Tubulin purified from Xenopus laevis and tropicalis eggs does not differ in the PTM pattern. DM1a recognizes α-tubulin, K40 recognizes acetylated tubulin, Tyr recognizes tyrosinated and Detyr detyrosinated tubulin, Poly-Glu recognizes linear glutamate chains of 4 and more residues.
In the context of this proposal, we will

- finalize the biochemical characterization of both tubulin proteomes by (quantitative) mass spectrometry, Western blot analysis and structural studies by cryo-electron microscopy.
- functionally link the differences in tubulin biochemistry to the observed differences of in vitro microtubule dynamics.
- test the contribution of different tubulins on spindle assembly and geometry by adding different tubulins to spindle reactions in Xenopus egg extracts.

These results will reveal the importance and function of tubulin heterogeneity. Although our system compares tubulin from two different species, the tubulin genes are very closely related and therefore will also give insight into the role of different isotypes within a single species, e.g., human. Furthermore, our work will reveal the role of post-translational modification on tubulin for spindle assembly. Both the regulation of tubulin isotypes and their post-translational modifications are suspected to be key for assembling the correct microtubule structure in the cell. Thus, our studies will not only reveal how assembly of the mitotic spindle works correctly, but also how its mis-regulation can lead to disease such as cancer.

AIM 2. MICROTUBULE BRANCHING AND SPINDLE GEOMETRY

The number of microtubules is mainly determined by microtubule nucleation. Although microtubule nucleation has been predicted to have a profound influence on spindle organization, there is no technique to directly visualize and measure microtubule nucleation in the spindle. It therefore remains unknown how different nucleation mechanisms, i.e., chromatin-mediated and microtubule-dependent microtubule nucleation, contribute to the overall spindle architecture. Research in the Petry lab has significantly improved our understanding of a specific type of microtubule nucleation: microtubule-dependent microtubule nucleation or microtubule branching (Petry 2011, Petry 2013). Microtubule branching is mediated via the 8-subunit protein complex Augmin (Goshima 2008, Lawo 2009) and stimulated by RanGTP and TPX2 (Petry 2013).

In a recent structure-function study, the Petry lab defined TPX2’s domain structure (Alfaro-Aco 2017) and identified a minimal fragment sufficient to stimulate branching in Xenopus egg extracts: TPX2 α5–α7. In many systems including C. elegans (Greenan 2010), Xenopus (Helmke 2014) and humans (Bird 2008), TPX2 has been shown to control spindle size. In the context of this proposal, we therefore will

- use the minimal TPX2 fragment to increase the number of microtubule branching in the spindle by add-aback and depletion experiments in Xenopus egg extracts.
• measure the effects of increased microtubule branching on spindle assembly and geometry, building upon studies of our recent studies (Reber 2013).
• estimate a rate of microtubule-dependent nucleation.

Because microtubules constitute the core of the mitotic spindle, it is clear that controlling the number of microtubules should have a profound effect on spindle geometry and function. However, up to now, approaches and tools have been missing to study this important biological question. With this aim, we will pioneer the study of how the number of microtubules influences spindle assembly and shape, and establish methods that can be applied to other factors that give rise to mesoscale microtubule structures in the cell.

AIM 3. MICROTUBULE BRANCHING AND LENGTH - SPINDLE GEOMETRY

Spindle microtubules have a distinct length distribution (Brugues 2011). Research in the Reber lab has significantly improved our understanding of intrinsic factors regulating spindle length in Xenopus egg extracts. Theoretical arguments of mass balance quantitatively link dynamic microtubule parameters, such as microtubule growth velocity and length, to phenomenological spindle parameters, such as length and shape (Reber 2013). Key to understanding how spindles are formed is to be able to define exactly when and where microtubules are formed, and along which trajectory they grow. In the context of this proposal, we will

• use different XMAP215 mutants (Reber 2013) together with the minimal TPX2 fragment to increase both, microtubule branching and microtubule length.
• test whether we can ‘saturate the system’ to confirm / exclude the hypothesis of tubular being the limiting component to spindle size.
• refine the mass balance model.

This aim will for the first time allow the modulation of two key parameters that determine spindle size, namely microtubule polymerization and nucleation. Taken together, we will use biochemical and biophysical methods, along with structural biology, to determine at the single molecule level how combinations of MAPs define the geometry and properties of microtubule patterns. This biochemical reconstitution will be combined with coarse-grained models to predict how these patterns are established. The ultimate goal is to reconstitute, in vitro and in silico, how substructures of the spindle are formed.
IMPACT

The goal of our team is to explain the way in which cells engineer larger scale structures. After the last century set the stage with advancing the sequencing of genomes and single molecule studies, time is now ripe to investigate how the many pieces of the puzzle make cell function. However, this goal is limited by how much a single laboratory can achieve and can only be reached via strategic partnerships. Recent important biological insights from our own work and from that of many others has underscored the power of interdisciplinary studies, combining cell biological methods with biophysical techniques and computational modeling. Even more important is form strong and productive collaborations, where the result is more than the sum of its part. We know that the most productive and impactful scientific advances on the fundamental problem of intracellular organization will come from expanding the frontiers of such collaborations, bridging international and disciplinary boundaries. Here, we describe a unique international collaboration that combines biochemical reconstitution, structural biology, biophysical measurements and modeling, and robust analysis of phenotypes at the cellular level. Insight into the physical principles that underlie the assembly of the spindle as a molecular machine is likely to have important implications for their function in cell growth, proliferation, and size control. This will not only explain how cell division works and life is propagated, but also uncover how cell division gets deregulated and how this contributes to disease, such as uncontrolled cell proliferation.
3. BENEFICIARIES

REBER LAB

The Reber lab uses cell-free biochemistry, single molecule biophysics, advanced light microscopy, and quantitative image analysis to elucidate the biochemical and biophysical principles that underlie the self-organization and scaling of subcellular organelles, in particular the metaphase spindle. In the context of this project proposal, the Reber lab will in particular study the contribution of different nucleation pathways on spindle length and shape. Although microtubule nucleation is predicted to contribute in setting both, spindle length and shape, the relative contribution of different nucleation pathways remains unknown. The effects of relevant proteins on (1) microtubule dynamics will be assayed by total internal reflection (TIRF) microscopy for single molecule methods and stop flow for bulk biochemistry assays and on (2) more general on spindle organization by using cell-free \textit{in vitro} reconstitution assays and confocal microscopy.

In addition, the lab has pioneered the purification of Xenopus tubulins. During the last 50 years microtubule biochemistry has mainly relied on native tubulin purified from mammalian sources, typically bovine or porcine brain. Because tubulin cannot be recombinantly expressed and its purification from non-animal sources has been extremely challenging, the biochemical characteristics of cell-type specific tubulins, such as present in Xenopus oocytes, have not been studied. Although tubulin is highly conserved in eukaryotes, interactions are likely to be affected when heterologous components are used in reconstituted biochemical assays. Based on our recently designed affinity purification, Xenopus tubulins can now be studied directly with much greater physiological relevance.

Previous experience relevant to the tasks:
- Protein biochemistry, enzymology, structure-function analysis
- \textit{In vitro} reconstitution of spindles in \textit{Xenopus} egg extracts
- Total internal reflection (TIRF) microscopy
- Confocal laser scanning microscopy

Relevant publications:
PETRY LAB

The Petry lab uses advanced light microscopy, biochemical and cell biological methods to examine the microtubule nucleation pathways that contribute to building the microtubule cytoskeleton. In parallel, the Petry lab employs electron microscopy and X-ray crystallography to determine the structural basis of key microtubule modulators involved. In the context of this project proposal, the Petry lab will in particular study the molecular components of that define patterns of the spindle with the goal to uncover their mechanism of action at an atomic level. Reagents and results from these biochemical and structural studies can in turn be used to reveal how these molecules drive pattern formation in the spindle and contribute to the mesoscale behavior that resembles a phase transition.

Previous experience relevant to the tasks:

- Total internal reflection (TIRF) microscopy
- Negative stain and Cryo Electron Microscopy (EM)
- X-ray crystallography
- Reconstitution of microtubule nucleation pathways in extract and in vitro

Relevant publications:

4. BENEFICIARIES TRAINING & DISSEMINATION

ANNUAL MEETINGS AND EXCHANGES DURING THE ACADEMIC YEAR

Our primary objective is to connect researchers at Princeton University and Humboldt University through a multi-faceted approach combining a series of exchanges and visits. One specific aspect of this will be a yearly meeting, which alternates between one of the partner institutions. This meeting will include PhD students and postdoctoral fellows from our labs, and provide opportunities for them to present their research results in talks, so that these young scientists will be able to establish international scientific contacts, and will be broadly educated about biophysical approaches to their ongoing research. This annual meeting will last for three days to provide sufficient time for discussion, and allow for additional meetings and interactions involving other researchers, with the goal of seeding other collaborations between Humboldt and Princeton. As the research interactions develop among our laboratories, we will encourage additional exchanges and visits between students and postdocs in our laboratories, to allow for deeper collaborations that can only be developed by working side-by-side for several weeks.

In addition, we will capitalize on our positive experience at the Marine Biological Laboratory in Woods Hole in 2016. We will send PhD students and postdoc from our labs to perform research together for a summer with little distraction in the most stimulating scientific environment of the world. This research stay will take place upon initiating research on our aims and will mostly be used to push projects forward in a short time frame.

VISITING RESEARCH FELLOWSHIP

The Petry and the Reber lab share tight bonds, which were mainly established during our stay at the joint summer lab at the Marine Biology Laboratory (MBL) in Woods Hole in 2016. We believe that joint teams are the most efficient way to improve communication and research between labs and thus link the scientific community in different universities. We therefore will each devote one lab member to work on this project proposal. These two lab members will be considered jointly mentored by Dr. Petry and Dr. Reber, which means that he/she will spend time in Princeton as well as in Berlin, depending on the technical needs and methodological requirements of the research project. By establishing joint mentorship, we hope to promote research cooperation and leverage synergies between the two universities but also to empower the two candidates for their next career step at a leading international research university. The additional option to grant a visiting research fellowship at either university will give us the flexibility to invite a (junior) researcher to spend time in our labs. The candidate junior researcher will complement the expertise in our labs, and thus help our
lab members working on the proposed project to raise their research to a whole new level. Potential candidate researchers will add most value by providing expertise in the fields of image analysis, soft active matter physics, and/or microfluidics. We believe that the combined strengths of two renowned research labs will attract highly promising and outstanding (junior) researchers.

5. FUTURE ENDEAVORS

The proposed work so far focuses on studying mechanistic principles of mitotic spindle formation and function. There are, however, many more non-membrane bound larger scale structures in a cell. The organizational principles of most of these organelles remain elusive. These future avenues together with the outcome of the current proposal will constitute the basis for a follow-up application for future funding. We intend to submit such an application during the second half of this project when initial data solidifies. Potential funding agencies and programs include:

- The National Science Foundation (NSF) and the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) fund collaborations in Natural and Engineering Sciences to support German-U.S. research and training projects.
- The Federal Ministry of Education and Research (BMBF) provides funding for projects in the fields of health research and life sciences. In addition, the BMBF division responsible for cooperation with the USA funds workshops and scientist exchanges as well as activities to support young researchers and prepare projects.
- The Great Generation Fund for Research and Education at the MBL supports scientists at all levels for summer research.
- The Stephen W. Kuffler Fellowship Fund is intended to encourage the career development of promising young investigators by helping to support them in the intense intellectual atmosphere of the MBL for the summer. It covers part of the costs of laboratory rental, housing, and other personal expenses.
- The Nikon Fellowship is available to a young investigator doing research in an area of biology in which she or he can make extensive use of advanced microscopy or micro-manipulation systems provided by Nikon, Inc. for their laboratory and also benefit from the technical expertise offered by Nikon, Inc. to support these instruments. This generous fellowship includes a summer laboratory, housing, and a budget for incidental expenses, equipment rental, and supplies.
- The Herbert W. Rand Fellowship provides funds to bring to the laboratory a distinguished investigator, preferably, but not necessarily, from outside of the United States. The award covers or assists with such expenses as travel, supplies, laboratory fees, and a stipend.