IN VITRO ASSEMBLY OF ARTIFICIAL MITOTIC SPINDLES
USING DIELECTROPHORESIS

A Thesis in
Bioengineering

by

Vidhya Aravamuthan

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The thesis of Vidhya Aravamuthan was reviewed and approved* by the following:

William O. Hancock  
Associate Professor of Bioengineering  
Thesis Co-Advisor

Richard J. Cyr  
Professor of Biology  
Thesis Co-Advisor

Peter J. Butler  
Associate Professor of Bioengineering

Herbert H. Lipowsky  
Professor of Bioengineering  
Head of the Department of Bioengineering

* Signatures are on file in the Graduate School.
ABSTRACT

The separation of duplicated chromosomes during mitosis is achieved by the mitotic spindle which consists of microtubules and associated motor proteins. Many motor proteins are known to interact with microtubules and affect their dynamics and organization. Knockout strategies have been used to probe the spindle in cells, but due to the inherent redundancy built into this complex process, deleting individual motor proteins often has only subtle effects. Single molecule studies have been used to study the properties of isolated motor proteins. However, they do not provide any information on the interaction of motors with complex microtubule assemblies like the spindle. The goal of this project is to develop novel engineering approaches for the study of mitosis that bridges single molecule studies and *in vivo* studies in cells. The approach is to assemble artificial spindles *in vitro* to study and characterize interactions of single motors with the microtubules that make up the spindle. To achieve this goal, we have used micro-electrodes fabricated on a quartz substrate. AC electric fields were then applied across the electrodes, which results in electroosmotic flows and dielectrophoretic forces. Microtubules assemble at the electrode tip due to the resultant electrokinetic forces and are then immobilized on the neutravidin patterned electrodes. These assembled microtubules were then extended with rhodamine tubulin to obtain overlapping opposed microtubules. The ability to extend microtubules in the flow cell facilitates the study of interesting phenomena like plus tip tracking of motor proteins and the influence of motors on polymerization kinetics. This assembly of microtubules then provides a platform to study the function of single motors and populations of motors and their effect on the spindle. The scope of this research can be further expanded by using beads coated with a single motor type in conjunction with optical traps to impose external forces. This novel experimental tool should provide important clues towards understanding force generation and microtubule rearrangements by molecular motors in the mitotic spindle.
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I would like to thank my husband Narayanan and the rest of my family who stood behind me at all times.
Chapter 1
Introduction

Cell division, a basic process of life, has been a subject of intense research, employing techniques ranging from imaging to deletion studies to biochemical studies. Cell division studies have implications in drug development, cancer treatment and fertility related problems. The key event in cell division is the segregation of sister chromatids into two daughter cells by attaching themselves to microtubules. Among the many motor proteins involved in cell division, kinesin and dynein play key roles in spindle morphogenesis and chromosome segregation. Motor proteins hydrolyze ATP and convert this chemical energy into mechanical work. Motor proteins are involved in many functions like microtubule destabilization (by Mitotic Centromere Associated Kinesin, MCAK) (Maney et al. 1998), in bundling of microtubules (by Arabidopsis thaliana kinesin, ATK5) (Ambrose et al. 2005), and being localized in the kinetochore area (like dynein and CENP-E) (Banks and Heald 2001).

Although the function of motor proteins have been studied by deletion studies in cells, there is a possibility that the cell could compensate for that loss of function by error correcting mechanisms, as in the case of ATK1 (Marcus et al. 2003). Mutants of ATK1 show abnormal microtubule accumulation and spindle polarity during early stages of mitosis which is eventually resolved and chromosomes are segregated normally due to the redundancy in the cells (Marcus et al. 2003). Single molecule studies have been used to
study the motor kinetics, but this does not give any information on the interaction of motor with a complex microtubule assembly like the spindle.

It is our endeavor to bridge between the study of motor proteins in cells and the study of motor proteins at the single molecule level. Our goal is to create an artificial spindle assembly (see Fig 1.1 for schematic) which will enable us to study the function of single motor proteins in the complex geometry of the mitotic spindle. In this thesis, we use engineering approaches like dielectrophoresis and protein patterning to assemble artificial mitotic spindles. This allows an opportunity to introduce single motor proteins and observe their interaction with the spindle and model spindle dynamics.

**Figure 1.1:** Artificial spindle schematic - The biotinylated minus ends (blue) of the segmented microtubule binds to the neutravidin patterned electrodes. The purple lines indicate rhodamine extensions in the electrode gap. Microtubules are assembled by dielectrophoresis and further immobilized at the electrode tip due to biotin-neutravidin link.
Background

Mitosis and Motor proteins

The mitotic spindle is a dynamic structure brought about by a series of microtubule growth and shortening events. These events are concurrent with the movement of chromosomes towards the equator and to the spindle pole. Before the start of mitosis, both genomic DNA and the centrosome (in animal cells) replicate. The start of mitosis, prophase, sees the movement of centrosomes to form the spindle pole. A tetrameric motor protein from kinesin-5 family, Eg5, is known to affect the separation of centrosome and null mutants of Eg5 have shown monopolar spindles (Zhu et al. 2005). RNA interference mediated depletion of other motor proteins like Klp10A, Klp61 and Klp67A form monopolar spindles as well, while depletion of the kinesin-14 motor, Ncd, causes multipolar spindles (Goshima and Vale 2003). Metaphase sees the alignment of chromosomes at the equator of the cell due to pushing forces. These pushing forces could be due to the polymerization of the microtubules and/or from various motors acting on the microtubule. It is seen that chromosome congression at the metaphase plate is ensured by many kinesins like CENP-meta, Klp3A and Nod (Goshima and Vale 2003). After metaphase, during anaphase, the chromosomes move away from the equator towards the spindle pole. This is achieved by both spindle pole movement and by microtubule depolymerization at both plus and minus ends (Rogers et al. 2004). MCAK, a motor involved in microtubule depolymerization, has been shown to be involved in chromosome separation during Anaphase (Maney et al.
In telophase, separation of chromosome is complete and the nuclear membrane forms around the separated chromosomes (see Fig 1.2).

Figure 1.2: Various stages of mitosis – (a) Centrosome movement in prophase (b) Movement of chromosomes to metaphase plate (c) Separation of chromosomes towards spindle pole during anaphase (d) Formation of nuclear membrane around the daughter chromosomes (Figure adapted from Schliwa 2003)

Research in microtubules and motors, apart from answering many questions from a pure science perspective, is significant due to its direct consequences in cancer and other diseases. Motors like KIF1A are involved in neurodegenerative disease and KIF5 in Alzheimer’s (Seog et al. 2004). Using our proposed artificial spindle, those proteins involved in division and specifically in spindle formation and maintenance like Eg5, MCAK and dynein (Banks and Heald 2001), can be studied individually.
Motor proteins

In this thesis the focus will be on two motors: conventional kinesin and ATK5. Of the 14 kinesin families, conventional kinesin is most well characterized. It is a plus ended motor and for every ATP hydrolyzed it takes one 8 nm step (Coy et al. 1999). It is a processive motor, i.e. it can walk on the microtubule for long distances (~1 µm) (Howard et al. 1989; Block et al. 1990). Every step kinesin takes is due to conformational changes in the head which is in turn tightly coupled to ATP hydrolysis cycle (Hancock and Howard 1999). Conventional kinesin consists of two heavy and two light chains. The heavy chain contains microtubule binding domain and a catalytic domain which in turn is connected to neck linker and coiled coil. The light chain is involved in cargo binding (Hirokawa et al. 1989).

While conventional kinesin is a processive plus ended motor with its motor domain at the N terminal, motors in kinesin-14 family protein have their motor domain at the C terminal and move towards the minus ends of the microtubule. Arabidopsis thaliana kinesins (ATK1, ATK2, ATK3, and ATK5) are minus ended mitotic motor proteins belonging to Kinesin 14A sub-family (Mitsui et al. 1993; Mitsui et al. 1994). ATK5 is 83% similar in sequence to ATK1, which is a non processive minus end directed motor (Marcus et al. 2002). ATK5 has been implicated in focusing of spindle pole and spindles and the authors suggest that it is due to +TIP action and microtubule bundling that it zippers the microtubules at the pole (Ambrose et al. 2005).
Current approaches used to study mitosis

A study that best mimics spindle assembly in vitro, has been conducted using Xenopus egg extracts (Heald et al. 1997). In this study, the authors assembled chromatin using DNA coated magnetic beads in the presence of Xenopus egg extract. They observed formation of nuclear envelope when DNA beads were incubated with extracts from interphase. Interestingly, in the presence of labeled tubulin, spindle-like structures were observed (see Fig 1.3.a) (Heald et al. 1997). The authors suggest three key step interactions of motors and microtubules lead to bipolar spindle formation (Fig 1.3.b). In the first step, nucleation, chromatin beads randomly interact with microtubules. In second step, dynein groups the minus ends forming a nearly linear bundle and finally the plus ends in the chromatin beads push the microtubules away.

Figure 1.3: (a) Bipolar spindle assembly in the presence of microtubules and Xenopus egg extracts and DNA coated beads, (b) Possible mechanism of spindle formation through a three step process (Figure reproduced from Heald et al. 1997).
Another *in vitro* study that assembles microtubules in various geometries uses oligomeric motors (Nédélec et al. 1997) (see Fig 1.4). In this study, the authors use both stabilized and dynamic microtubules along with oligomeric motors. The relative concentration of microtubules and motors were varied in closed chambers and the various structures formed are observed using dark field microscopy. The authors report formation of spindle-like geometries on incubating the microtubules with both plus and minus end directed motors.

**Figure 1.4:** Microtubule geometries formed by addition of multiheaded kinesins to stabilized microtubules (a) Combination of asters and vortices, (b) Asters, (c) bundles (d), vortices formed for different microtubule to motor ratios. (Figure reproduced from (Nédélec et al. 1997)).

Depletion studies in cells have also been undertaken to study interaction of various motors with microtubules. Goshima and Vale (2003) studied the function of different motors by observing the effects on mitosis on depleting motors by RNA interference. They found many redundant or error correcting mechanisms in the cell when a single motor was depleted. For example, four motor proteins - Nod, CENP-meta, Klp3A and Klp67A are involved in alignment of chromosome during metaphase. Hence, it is difficult to study the effect of one motor by depletion studies.
Manipulation of Microtubule organization in vitro

Efforts to immobilize microtubules in certain geometries are being driven by the desire to achieve efficient transport in microfluidic systems. Currently, efforts towards immobilizing microtubules in a particular geometry have been achieved by fabricating open and closed microchannels (Moorjani et al. 2003; Huang et al. 2005). Microtubules have been magnetically functionalized and then aligned by magnetic fields (Platt et al. 2005). An elegant method to control the microtubule alignment is by using dielectrophoresis (Uppalapati et al. 2008). In this technique, the polarized microtubule moves according to the forces generated in the flow cell due to the application of AC electric fields (Uppalapati et al. 2008). The details of this phenomenon will be discussed in the following section.

This thesis is built on earlier work done in the Hancock lab using AC electrokinetic mechanisms such as dielectrophoresis, electroosmotic flow to align microtubules. After aligning the microtubules, they will be immobilized by biotinylating the microtubules and binding them to neutravidin coated electrodes.
**Dielectrophoresis**

Microtubules are protein polymers with a net negative charge and hence applying electric fields to separate or transport or assemble them is possible as has been shown in the Hancock lab (Jia et al. 2004; van den Heuvel et al. 2006; Kim et al. 2007). However, applying DC field causes electrolysis of the electrolyte and bubble generation leading to electrode damage. Applying non-uniform AC electric fields is another option to transport microtubules.

Dielectrophoresis is the movement of a polarizable particle in the presence of an electric field. If a particle is more polarizable than its medium, then it moves towards high field regions leading to positive dielectrophoresis. Negative dielectrophoresis occurs when a particle is less polarizable than the medium, leading to movement towards low electric field regions (Fig 1.5) (Morgan et al. 2003).

![Figure 1.5: Positive (grey particles) and negative (black particles) dielectrophoresis in a castellated electrode (Figure reproduced from Morgan et al. 2003)](image)
**Electroosmotic flows**

When a charged surface comes in contact with an electrolyte, opposite charges (counter ions) accumulate near the surface of the electrode (see Fig 1.6). The Stern layer is the region of tightly associated counter ions and the diffuse layer is the region adjacent to Stern layer and is part of the bulk solution (Morgan et al. 2003).

![Formation of double layer, ions of opposite polarity on electrode surface](image)

**Figure 1.6:** Formation of double layer, ions of opposite polarity on electrode surface (Figure reproduced from Morgan et al. 2003).

In the presence of an electric field, these ions move due to the tangential force (see Fig 1.7.a). Fig 1.7.b shows the bulk flow generated by the movement of ions in the double layer. In the other half cycle (refer to Fig 1.7.a), a positive potential is applied to the left electrode and negative ions accumulate on the electrode. Ions move in the same direction as before since direction of tangential component of electric field remains the same.
Electroosmotic flows are frequency dependent and work well in intermediate frequencies (for the electrodes we used it was ~ 2.5 MHz). At high frequencies, since ions cannot accumulate in half cycle period, there is no electroosmotic flow (Morgan et al. 2003). At very low frequencies, there is no electroosmotic flow due to potential drop across the double layer resulting in zero tangential component acting on the double layer.

![Image](image_url)

**Figure 1.7:** (a) Forces acting on the double layer due to the applied electric field (b) Electroosmotic flow due to the movement of double layer due to tangential component electric field (Figure reproduced from Morgan et al. 2003)

**Electrothermal flow**

Although electroosmotic flows are maximal at intermediate frequencies, electrothermal flows are frequency independent (Morgan et al. 2003). Electrothermal flows are generated due to temperature gradients in the flow cell. In our experiments, the main cause of heating is due to irradiation from the fluorescence lamps, which is absorbed by the semi-opaque chrome electrodes. Fig 1.8 shows a simulated electrothermal flow profile. It is apparent from the figure that electrothermal flow is bulk phenomena whereas electroosmotic flow is a surface flow. Also note that the flow is towards the electrode gap, unlike the electroosmotic flow which is away from the electrode gap.
Hence, for our application of spindle formation, it is necessary to cancel out electrothermal and electroosmotic flow so that microtubules can accumulate on the electrode due to dielectrophoresis (DEP). DEP experiments were performed at 5MHz when electroosmotic and electrothermal flows cancel out each other (Uppalapati et al. 2008).

**Figure 1.8**: Electrothermal flow simulated for one of the ‘two electrode’ system at electrolyte conductivity of 2.1 mM m\(^{-1}\) and value shown in the box is the maximum velocity (Figure reproduced from Morgan et al. 2003).

**Thesis Overview**

Mitosis is a complex phenomenon and many motor proteins are involved in spindle formation and maintenance. We aim to create an *in vitro* environment with microtubule organization resembling those found in mitotic spindles. So far, Maruti Uppalapati (from the Hancock lab, Pennsylvania State University) and Ying-Ming Huang (from Jackson lab, Pennsylvania State University) have laid the groundwork by aligning microtubules using AC electrokinetic techniques (Uppalapati et al. 2008). They have also patterned
neutravidin for selective microtubule immobilization (Huang et al. 2008). In addition, new electrode samples for this work have been provided by Raymond Fok (from Jackson lab, Pennsylvania State University). In this thesis, we combine the above techniques to obtain artificial spindles by using segmented microtubules that contain biotinylated segments on their minus ends. We show the formation of artificial spindle and also the possibility of growing the microtubules in the flow cell. We also show that the age of the electrode affects microtubule binding.
References:


Chapter 2
Microtubule bundling in vitro by ATK5

Introduction

Kinesins are motor proteins with specific domains for microtubule binding, ATP hydrolysis, dimerization and cargo binding. Although kinesins are structurally diverse, certain key structural domains define their functions. The presence of the motor domain at the N- or C- terminal dictates the movement of the kinesin to plus or minus ends of the microtubule respectively (Vale and Fletterick 1997). Motors like Non claret disjunctional (Ncd) and the Arabidopsis thaliana kinesins ATK1, ATK2, ATK3, and ATK5 are members of the kinesin-14 family and are C-terminal motors that translocate towards the minus end of the microtubule (Mitsui et al. 1993; Mitsui et al. 1994).

Some Arabidopsis thaliana kinesins have their motor domain at the C terminal which is then the primary microtubule binding site. The secondary microtubule binding site for ATK2 and ATK3 is at the tail domain which is at the N terminal (Jiang et al. 2007). ATK1 and ATK5 share high sequence similarity with ATK2 and ATK3 (Jiang et al. 2007).

ATK5 is a minus ended motor which localizes to the growing plus tip of the microtubule i.e. ATK5 is a plus tip tracker (Ambrose et al. 2005). This plus tip localization is independent of the motor domain i.e. tail and stalk region is sufficient for plus tip activity. It has been seen that EB1, a plus tip tracker, attaches selectively to the growing
ends of a microtubule and not to a stabilized microtubule (Mimori-Kiyosue et al. 2000). In vitro studies using purified components of Mal3 (EB1 homologue), kinesin Tea2, Tip1 (Clip170 homologue) showed that Mal3 selectively binds to the growing plus tip and not to the lattice and growing minus ends (Bieling et al. 2007). Mal3 (or EB1) can associate with the growing plus tip either by co-polymerizing (i.e. binds to free tubulin in solution) or due to some structural difference of the microtubule at the plus end. Bieling and colleagues found that EB1 does not localize to the plus tip due to co-polymerization with tubulin. Hence, the plus tip activity is due to the structural difference of the microtubule at the tip either due to the presence of GTP-tubulin or due to presence of open ended structures like the protofilament (Bieling et al. 2007).

It is seen that null mutants of ATK5 not only have wide spindle midzone and poles during metaphase and anaphase, but also elongated and bent spindles during prometaphase. This widening of spindles has been attributed to the lack of bundling activity in ATK5 null mutants. It has been proposed by Ambrose and Cyr (2007) that ATK5 uses both its plus-tip tracking ability and minus-ended motility to capture antiparallel microtubules in the spindle and slide them across to decrease the lateral distance. This interaction also creates inward forces preventing elongation of the spindle and further straightens the spindle. The bundling ability of ATK5 implies two microtubule binding sites like another kinesin-14 motor, Ncd, which has an ATP dependent binding site at the motor domain and an ATP independent binding site at the tail (Karabay and Walker 1999). Tail of Ncd is positively charged and it electrostatically interacts with the negatively charged E-hook of the microtubule (Furuta and Toyoshima
2008). The nature of the ATK5 tail, however, is not very well understood. It could either interact with the microtubule through a plus tip protein complex or directly bind to the microtubule plus end. ATK5 could electrostatically interact with the microtubule like Ned (Furuta and Toyoshima 2008) and diffuse one dimensionally or bind tightly to the microtubule.
Materials and Methods

The protocol for the following experiments were developed by Christian Ambrose from the Cyr lab (Ambrose and Cyr 2007). Arabidopsis thaliana Kinesin 5 (ATK5) and Drosophila melanogaster kinesin heavy chain (KHC) were expressed in E.Coli BL21(DE3) cells. Cell lysates of ATK5 and KHC were obtained as described previously (Marcus et al. 2002). The transformed bacterial cells were grown overnight and expression induced by adding 0.5 mM isopropyl beta D-thiogalactoside (IPTG). The cells were then centrifuged at 5000 rpm and resuspended in a buffer (containing 1 mM MgATP, 1 mM PMSF, 20% glycerol, 25x protease, 5 mM $\beta$-Mercaptoethanol (BME)) in PM buffer (50 mM PIPES, 1 mM MgSO$_4$, 1 mM EGTA, pH 6.9). The cells were sonicated 2 times for 10 secs and centrifuged at 60,000 rpm for 30 mins to remove any insoluble components. The clarified supernatant was then used in these bundling assays.

A microtubule mix was prepared from Microtubules (MT). Three concentrations of microtubules were used - 0.13 µM, 0.2 µM and 0.5 µM (1:4 rhodamine to PC tubulin) along with 5 mM MgATP, 0.05 mM Taxol, 0.008 mg/ml catalase, 0.06 M glucose, 0.24 mg/ml glucose oxidase, 1% BME in PM buffer (50 mM PIPES, 1 mM MgSO$_4$ and 1 mM EGTA, pH 6.9). 10 µl of cell lysate was mixed with 20 µl of MT mix and flowed into a flow cell. Microtubule-motor interactions were observed using Zeiss Axiovert 8100TV inverted microscope (100x, 1.3 NA oil objective). Microtubules were imaged using Q-Imaging Retiga-SRV camera. The images were recorded using ImagePro Plus 6.0.
Results and Discussion

ATK5 is similar in amino acid sequence to ATK2 and ATK3 and is hypothesized to bind to microtubule at the tail domain (Jiang et al. 2007). In order to test the hypothesis that ATK5 bundles microtubules *in vitro*, ATK5 was expressed in bacterial cells and the bacterial cell lysate was used in bundling assay. A mix of microtubules and ATK5 with ATP was introduced into a flow cell. Fig 2.1 shows fluorescent images of the structures formed for three different microtubule concentrations of ATK5 and KHC. ImageJ analysis of intensities of bundles of microtubules and individual microtubule was done and the number of microtubules involved in a bundle was determined. This analysis was done for all the bundles in a field of view. Finally, a distribution for the number of microtubules in a bundle is plotted in Fig 2.2.

*ATK5 bundles microtubules in vitro*

As seen from Fig 2.1, in the presence of ATP as the ratio of microtubule to motor concentration is varied, the microtubule geometry changes from bundles to asters. The number of microtubules incorporated into a bundle also varies with the microtubule to motor ratio. At low microtubule concentration (for fixed ATK5 concentration), almost all the microtubules are involved in small microtubule bundles (≤5 microtubules/bundle). At high microtubule concentrations (0.5 µM) for ATK5, there are small bundles, but almost all the microtubules are involved in asters (see Fig 2.1.a). At low microtubule concentrations (for fixed KHC concentration), there is no definite microtubule structure.
At high microtubule concentration (0.5 µM) for KHC, almost all the microtubules are involved in bundles (see Fig 2.1.b).

**Figure 2.1:** Microtubule geometry for different microtubule concentration in the presence of ATP a) 0.5 µM MT with ATK5 b) 0.5 µM MT with KHC c) 0.2 µM MT with ATK5 d) 0.2 µM MT with KHC e) 0.13 µM MT with ATK5 f) 0.13 µM MT with KHC

Microtubule geometry varies with the change in microtubule concentration as seen above in Fig 2.1. Next, we wanted to determine if the bundle size changes with the microtubule
concentration. For every condition (i.e. microtubule concentration and motor type), the intensity of the bundle was compared to the intensity of a single microtubule to calculate the number of microtubules in a bundle. This was done for every bundle in the field of view. This analysis gave a distribution for the number of microtubules in a bundle for every motor type and motor concentration as plotted in Fig 2.2. At low microtubule concentrations (0.13 µM and 0.2 µM Microtubules), most of the microtubules are involved in small microtubule bundles (≤ 5 microtubules in a bundle). As microtubule concentration is increased to 0.5 µM (for both ATK5 and KHC), the number of small bundles reduces and number of larger bundles (≥ 5 microtubules in a bundle) increases.

Figure 2.2: Distribution of the number of microtubules in a bundle for every motor type and concentration. Fluorescence intensity of every microtubule bundle and single microtubule was measured using ImageJ to obtain the number of microtubules in a bundle.

Combining the data from Fig 2.1 and Fig 2.2 we obtain Table 2.1, which gives the distribution of microtubule structures formed by ATK5 and KHC. It can be seen that
KHC does not form asters for the given microtubule concentrations (0.13 µM, 0.2 µM, 0.5 µM). Whereas at high microtubule concentration (0.5 µM), about 77.3% of the microtubules are involved in bundles. ATK5, on the other hand, forms both bundles and asters. For ATK5, as the microtubule concentration is increased from 0.13µM to 0.2µM and 0.5µM, microtubules form bigger bundles and asters. The density of aster increases with the increase in microtubule concentration i.e. from 0.2 µM to 0.5 µM. The percentage of microtubules involved in bundles falls from 87.5% for 0.13 µM microtubules to 75% for 0.2 µM and 76.3% for 0.5 µM microtubules since microtubules are also involved in the formation of asters.

<table>
<thead>
<tr>
<th>Microtubule Structure</th>
<th>0.13µM MT ATK5</th>
<th>0.13 µM MT KHC</th>
<th>0.2 µM MT ATK5</th>
<th>0.2µM MT KHC</th>
<th>0.5 µM MT ATK5*</th>
<th>0.5µM MT KHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of Microtubules in bundles</td>
<td>87.5%</td>
<td>--</td>
<td>75.0%</td>
<td>--</td>
<td>76.3%</td>
<td>77.3%</td>
</tr>
<tr>
<td>Percentage of Microtubule in Asters</td>
<td>--</td>
<td>--</td>
<td>Yes</td>
<td>--</td>
<td>Yes</td>
<td>--</td>
</tr>
<tr>
<td>Unstructured geometry</td>
<td>--</td>
<td>Yes</td>
<td>--</td>
<td>Yes</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
Aster Formation

In a simulation study done by Surrey and colleagues, a multimeric motor is modeled to be either in free solution or bound to one microtubule or, bound to two microtubules exerting a force on both (Surrey et al. 2001). The simulation study found an inverse correlation between the processivity of a motor and intensity of the aster formed. Also other kinetic parameters such as on and off rates can influence this geometry. We can see from our observations that although KHC is a processive motor, asters are not formed for the given concentrations. However, the above mentioned study simulated oligomeric kinesins, while we used only KHC, which has a microtubule binding domain in the tail (Andrews et al. 1993).

Contrastingly, ATK5 which has 83% sequence similarity with ATK1 (Ambrose et al. 2005) formed asters. ATK1 is a minus ended non processive motor (Marcus et al. 2002). Hence, it is reasonable to assume that ATK5 is a non-processive motor. In studies done with non-processive Ncd, it has been found that they act cooperatively (Higuchi and Endow 2002) i.e. when one motor unbinds other Ncd motors bind to the microtubules pushing the microtubule forward (in a gliding assay). Similarly, in this bundling assay, it is possible that the aster formation is due to the cooperativity of ATK5 wherein many ATK5 motors are localized in the aster. Also, the fact that asters are formed in vitro suggests that asters could be formed in vivo too.
**Bundling of Microtubules**

In our experiments we see that KHC bundles microtubules *in vitro* at high microtubule concentrations (0.5 µM). Conventional kinesins have been shown to bundle microtubules *in vivo*. An *in vivo* live imaging study on fungus *Ustilago maydis* confirmed the role of conventional kinesin in microtubule bundling (Straube et al. 2006). Their study also indicated that 25% of the times up to 3 microtubules were involved in a bundle which were predominantly straight and only 6% of them were bent (Straube et al. 2006). It has been found that, kinesin in its inactive state folds over to bury the tail domain where the IAK domain in the tail interacts with the head (Hackney and Stock 2000). Kinesin truncated to 960 amino acids (a.a.) folds over due to the presence of IAK domain whereas, truncates till 945 a.a. and 937 a.a. do not have IAK domain leading to unfolding of kinesin molecule and to microtubule binding (Hackney and Stock 2000). The tail of kinesin binds to the microtubule tightly, possibly due to electrostatic interactions with the microtubule.

Conventional kinesin is a plus end directed motor, whereas Ncd and ATK5 are minus end directed motors from the Kinesin-14 family. Ncd has two microtubule binding sites and is known to bundle microtubules *in vivo* through the tail domain (Oladipo et al. 2007). There is a positively charged K-rich region in the tail domain that electrostatically interacts with negatively charged E-hook region of the microtubule (see Fig 2.3) (Furuta and Toyoshima 2008). This electrostatic interaction of Ncd with the microtubule makes Ncd a processive motor under low ionic strength buffers (Furuta and Toyoshima 2008).
ATK5 is known to be a plus end tracker i.e. it remains preferentially attached to plus end of a growing microtubule tip (Ambrose et al. 2005). This preferential attachment to a growing end is through the interaction of the tail/stalk region of ATK5 with the microtubule and not through motor domain. ATK5 has also been implicated in focusing of spindle pole. This is due to the zippering action of ATK5 caused by plus tip
localization and motor movement towards the minus end. The authors propose that ATK5 can preferentially bind either directly to the microtubule or through a +TIP complex (Ambrose et al. 2005). Motor proteins can interact with the growing plus ends by either of three mechanisms – active transport to the plus ends by a motor, affinity for the microtubule end structure or by attaching to free tubulin in solution and co-polymerizing to the microtubule end (Cassimeris 2007). From our observations, we see that ATK5 bundles taxol stabilized microtubules. Hence, it is reasonable to conclude that the interaction of the motor to the microtubule is not due to any of the plus tip mechanisms. Rather it is due to direct binding of the motor to the microtubule.

Fig 2.4 (a, b, c) below indicates possible scenarios when ATK5 binds tightly and loosely to the plus end. If the tail of the ATK5 is loosely bound to the microtubule (like the electrostatic interaction seen in Ncd (Furuta and Toyoshima 2008)), then we can expect the microtubules to remain straight (see Fig 2.4.b). This is because the motor can translocate and bundle the microtubule. If ATK5 is bound to the plus tip tightly through a protein complex localized at the plus end, then we can expect to see curled microtubules (see Fig 2.4.c.). This is due to anchoring of ‘tail’ end of the motor on a microtubule while the ‘motor’ end of the motor moves on another microtubule. What we observe are predominantly straight microtubules and bundling of taxol stabilized microtubules. Hence it is reasonable to conclude that there is weak but direct interaction between the motors and microtubules.
Figure 2.4. Possible scenarios of ATK5 binding to the microtubule (a) ATK5 starts to walk (bundle) (b) Weak direct binding of motor to the microtubule (c) Tight plus tip binding of ATK5.
References:


Chapter 3

Spindle assembly in vitro using Dielectrophoresis

Introduction

In order to exploit the microtubule-motor combination in nanotechnology, there are two main approaches: (i) immobilize motors on the surface and let the microtubules localize themselves, or (ii) immobilize microtubules on the surface and let the motors localize themselves. This selective immobilization is done in order to harness their potential to do directed work. The first approach of selectively immobilizing motors has been achieved by coating a substrate with Polymethylene methacrylate (PMMA) and forming tracks by UV photolithography. Myosin motors were then seen to adsorb only to PMMA tracks and actin motility was observed only on the PMMA tracks (Suzuki et al. 1997). Microtubules similarly have been shown to move along the axis of polytetrafluoroethylene (PTFE) surface functionalized with kinesin (Dennis 1999). Apart from this, lithographically patterned microchannels and rectifiers have been shown to restrict the motion of microtubules (Moorjani 2003; Jia et al. 2004; Huang et al. 2007).

The second approach of selectively immobilizing microtubules has been achieved using electric fields, magnetic fields and fluid flow. Microtubules have been functionalized with magnetic particles using biotin-neutravidin interaction and aligned in magnetic fields (Platt et al. 2005). However, use of this sort of alignment for creating artificial spindle is limited due to the reduction of kinesin-microtubule interaction owing to bulkiness of neutravidin. This issue has been addressed using segmented microtubules,
allowing greater probability of kinesin to interact with unlabeled microtubule segment (Hutchins et al. 2007). Another approach has been to use fluid flow to align microtubule seeds which then subsequently interact with the surface (Brown 2002).

For our goal of creating an artificial spindle, we need to assemble an array of anti-parallel microtubules such that the plus ends of the microtubules face each other. Minus ends of the microtubules needs to be secured and be farthest away (see schematic in Fig 3.1). To achieve this end, we build this work on an earlier work on dielectrophoresis and neutravidin patterning (Huang et al. 2008, Uppalapati et al. 2008). In this work by (Huang et al. 2008) flow cells were formed on top of chrome electrodes and neutravidin was adsorbed in the flow cell area. This neutravidin was patterned by irradiating the backside of the quartz sample with UV. This ensured that neutravidin was present only on the electrode (Huang et al. 2008). They were also successful in assembling microtubules at the tip of two opposing electrodes by dielectrophoresis (DEP) (Uppalapati et al. 2008).

![Schematic of artificial spindle formed on neutravidin coated electrodes and segmented microtubules (Cy5 seeds (blue) and rhodamine extensions (purple))](image)

**Figure 3.1:** Schematic of artificial spindle formed on neutravidin coated electrodes and segmented microtubules (Cy5 seeds (blue) and rhodamine extensions (purple))
In the present work, we use dielectrophoresis and neutravidin patterning to assemble and bind biotinylated microtubules at the electrode tip. We study the kinetics of microtubule assembly by taking successive images of spindle assembly and analyzing them off-line. We have optimized the patterning process for microtubule assembly. Finally, in order to obtain overlapping microtubules at the electrode gap, we extend the microtubules in the flow cell.

Even though microtubules assemble at the electrode tip when the field is on, binding of the microtubules is poor when the field is switched off. This is probably due to the erosion of the electrode with repeated use or due to accumulation of debris. This microtubule binding problem was solved by using new electrodes. With new electrodes we were able to obtain good microtubule binding and extension. Microtubule assembly followed a linear increase with time.
Materials and Methods

Preparing microtubules

Purified tubulin was obtained from bovine brains (Williams and Lee 1982). The tubulin was then labeled with rhodamine, biotin and Cy5 as described previously (Hyman 1991). N-ethylmaleimide (NEM) labeled tubulin binds to the minus ends of microtubules, blocking polymerization from the minus ends but does not affect plus end polymerization (Phelps and Walker 2000). To prepare NEM tubulin, 62.9 µM cycled tubulin was incubated with 2.5 mM NEM and 1.25 mM GTP on ice for 10 mins. The reaction was quenched with 14 mM β-Mercaptoethanol. Biotin tubulin was mixed with Cy5 tubulin in a 1:4 ratio and polymerized for 20 mins at 37°C along with 4 mM MgCl₂, 1 mM GTP, 5% DMSO in BRB80 buffer (80 mM PIPES, 1 mM MgCl₂ and 1 mM EGTA). The biotin seeds were then diluted 100 fold along with 10 µM Paclitaxel. 150 µl of biotin seeds were spun for 10 mins in Beckman Airfuge at 300 psi. Spun seeds were then resuspended in a solution containing 1.25 mM GTP, 5% DMSO, 4 mM MgCl₂, 6.29 µM NEM tubulin and RTU (40 µM of 1:4, rhodamine tubulin:cycled tubulin). This solution was incubated at 37°C for 20 mins and stabilized with 10 µM Paclitaxel.

For the extension experiments, biotin seeds as were immobilized on the electrode using DEP (Uppalapati et al. 2008). The seeds were then extended with an extension solution (RTU (10 µM of 1:4, rhodamine tubulin:cycled tubulin), 1.25 mM GTP, 5% DMSO, 4 mM MgCl₂) in BRB80 (80 mM PIPES, 1 mM MgCl₂ and 1 mM EGTA).
Sample Preparation

The electrode samples were cleaned in Piranha (1:4 Hydrogen peroxide : Sulfuric acid) for 10 mins. They were then exposed to UV-Ozone for 5 mins. They were then treated using 5% 3-aminoproyltriethoxysilane (APTES) for 30 mins. The hydroxyl groups on the quartz surface form a covalent bond with APTES by displacing the alkoxy groups. The sample was then washed with acetone and DI water and then blow dried. 30 µl of 5% gluteraldehyde was placed on top of the active electrode region and an 18 mm coverslip was placed on top of the gluteraldehyde forming a squash cell (approx 30-40 µm high) for 15 mins. The aldehyde groups on gluteraldehyde crosslink amine groups on the neutravidin and APTES, thus stably crosslinking neutravidin to the glass surface. After washing the sample with DI water and drying it, neutravidin was similarly incubated for 15 mins under a squash cell. The sample was then washed in a bath containing BRB6 and dried in a bath containing acetone for 30 secs and finally blown dried. Neutravidin was patterned by exposing the backside of the quartz sample with deep UV in the presence of Nitrogen for 110secs using the electrodes as the mask (Huang et al. 2008).

Dielectrophoresis experiment

A flow cell was created on top of the electrodes and 0.5 mg/ml casein was introduced for surface blocking. The microtubule solution containing 20 mM glucose, 0.02 mg/ml glucose oxidase, 0.008 mg/ml catalase, 0.5 mg/ml casein, paclitaxel (10µM), 0.35 M β-Mercaptoethanol and segmented microtubule was introduced into the flow cell. A 5MHz
field was applied across the electrodes (BK Precision, CA) (Uppalapati et al. 2008). Spindle formation was observed using Nikon eclipse E600 microscope (60x, water immersion objective). The images and videos were taken using Photometrics cascade 512B camera and MetaVue Imaging software.
Results and Discussion

In order to construct artificial spindles, we used dielectrophoresis to assemble microtubules and patterned electrodes to selectively bind the microtubules to the electrodes. The electrode surface was selectively coated with neutravidin by backside exposure of the quartz sample to UV (Huang et al. 2008). We made segmented microtubules by growing extensions of rhodamine tubulin from the Cy5-biotinylated seeds which are capped at their minus ends by NEM tubulin. The segmented microtubules were then injected into a flow cell and then the electric field was turned on and the accumulation was visualized under Nikon fluorescence microscope. Fig 3.2 shows the accumulation of segmented microtubules at the tip of the electrode when an AC field (5MHz, 22V) was applied to the electrodes.

![Figure 3.2](image)

**Figure 3.2:** Accumulation of segmented microtubules at the tip of the electrode when AC field is applied to the electrodes. Cy5 biotin seeds are in *green* and rhodamine extensions are in *red*
Spindle assembly

If the microtubules assembled in the right polarities then we would see (green) Cy5 seeds at the electrode tip and (red) rhodamine extensions at the gap. However, we can see from Fig 3.2 that it is difficult to distinguish between the polarities of microtubules when the field is on. The biotin-Cy5 seeds are capped at their minus end with NEM tubulin and hence the rhodamine extensions which are seen in Fig 3.3 and Fig 3.4 are from their plus ends only. When the field is turned off, we can be almost sure to obtain only plus ends at the gap. Since the whole flow cell is coated with casein, only those microtubules that have biotin at their tip remain bound to the neutravidin coated electrode. However, we observe that relatively shorter microtubules remain bound at the electrode tip when field is turned off (see Fig 3.3).

Figure 3.3: Spindle assembly on neutravidin coated electrodes formed by DEP. Biotin seeds labeled with Cy5 are in red and rhodamine tubulin extensions are in green.
One way to quantify and confirm the formation of spindle is by measuring the intensity of Cy5 seeds and rhodamine extensions. Ideally, if the segmented microtubules immobilized on the electrodes with minus ends on the electrode and plus ends at the gap, then we expect to see Cy5 intensity peak near the electrode followed by rhodamine intensity peak. Intensity distributions of rhodamine and Cy5 were obtained from ImageJ by drawing a line along the length of the electrode through the gap and measuring intensities along the line (Fig 3.4). Intensities were normalized to the peak intensity value and background noise was subtracted for each filter.

**Figure 3.4**: Intensity of rhodamine extension and cy5 seed along the electrode gap. The fluorescence intensity of Cy5 (blue) and Rhodamine (purple) were normalized to the peak intensity value and corrected for background noise. Gap between the electrodes is 20 µm.
The profile of fluorescence intensity of Cy5 tubulin shows an intensity peak near the electrode indicating the presence of seeds located near the electrode (Fig 3.4). The intensity distribution of rhodamine is wider near the electrodes compared to Cy5 intensity distribution, indicating a longer rhodamine extension compared to the Cy5 seeds. We see overlapping Cy5 and rhodamine intensities indicating non uniform distribution of seed and extension lengths. Overlap is probably due to rhodamine extensions having very small seeds. Thus, we have shown that spindle can be assembled with minus ends at the electrode and plus ends near the gap.

**Spindle kinetics**

In order to measure the spindle assembly kinetics, video of microtubule accumulation was recorded as soon as the electric field was applied to the electrodes. The kinetics of spindle assembly was obtained by measuring the intensity of microtubules assembled at the electrode tip. This was done by measuring the fluorescence intensities along the line drawn at the electrode tip using ImageJ. Fig 3.5.a shows a montage of microtubule assembly at various time points.
Figure 3.5 (a) Snapshot of microtubule accumulation on the electrode at different times (rhodamine filter). Each frame is separated by 19.9 seconds. (b) Accumulation of microtubule at the electrode tip is observed as soon as the field is turned on. The Y axis shows relative fluorescence intensities.

Microtubule accumulation at the electrode tips was quantified by measuring the intensity of fluorescence (microtubule accumulation) for series of images using ImageJ. The fluorescence intensity, which is a measure of number of microtubules, is plotted in Fig 3.5.b indicating assembly kinetics of microtubules. Electroosmotic and electrothermal flows as well as DEP forces are generated as soon as the AC electric field is applied (Uppalapati et al. 2008). Hence, it is expected that microtubules will accumulate at the electrode tip linearly with time as soon as the electric field is applied. However, we see a small time lag of 5 secs as soon as the field is turned on. This could be the time the
microtubules take to accumulate near the electrode surface from the bulk solution. As the microtubules accumulate on the tip of the electrode we expect to see the fluorescence intensity to level due to depletion of microtubules from the solution and/or crowding of microtubules at the electrode. We do observe a moderate plateau after 110 secs indicating saturation of microtubule accumulation on the electrodes. Although, antifade was used along with the microtubules, the effect of photobleaching cannot be ruled out and hence it possible the plateau seen in Fig 3.5 is due to photobleaching of the fluorophores. But frankly, we are do not fully understand the plateau.

**Age of the electrode**

Although microtubules are efficiently assembled at the electrode tip by DEP forces, many microtubules diffused away from the electrode once the electric field is turned off (Fig 3.6). This could be due to the presence of non-segmented microtubules and/or non-biotinylated seeds. Non-biotinylated seeds and non-segmented microtubules are attracted by DEP to the electrode, but they do not bind to the neutravidin coated electrodes. Biotinylated microtubules can also diffuse away due to insufficient neutravidin densities on the electrodes.
To understand this unbinding issue, we did control experiments on glass slides. Samples were coated with APTES followed by gluteraldehyde and then neutravidin. To check for effect of neutravidin treatment, we compared binding between dried neutravidin surface and un-dried neutravidin surface. Effect of patterning was studied by exposing part of the neutravidin coated glass slide to UV by covering the other half with a quartz coverslip. Binding was studied between the exposed and the unexposed regions of the control sample. We also checked for binding differences between the biotin seeds and the segmented microtubules on dried neutravidin coated glass surfaces. Although the number of bound segmented microtubule (35 microtubules per screen) is slightly less than the number of biotin seeds (50 microtubules per screen), it could be due to non-extension of
some seeds. Binding of segmented microtubules on acetone dried neutravidin surface also
did not vary much from non-dried neutravidin surface. Patterning neutravidin also did not
affect the microtubule binding (Table 3.1). However, we did see considerable difference
in microtubule binding between new and old quartz sample (see Fig 3.7).

Table 3.1: Some control experiments to decide the most important factor in spindle
formation (Results are in Number of Microtubules/screen)

<table>
<thead>
<tr>
<th>Sample preparation process</th>
<th>Control</th>
<th>With the process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drying with acetone</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>Patterning under UV</td>
<td>17</td>
<td>11 (Unexposed electrode region)</td>
</tr>
</tbody>
</table>

This difference in binding between old and new electrodes (Fig 3.7) could be due to
insufficient binding of neutravidin to the electrodes. In turn, neutravidin binding could be
affected by electrode surface roughness due to pitting. Neutravidin binding could also be
affected either due to debris on the electrode or due to electrode thinning (due to
electrolysis). Hence, an important factor determining binding is the age of the
quartz/electrode sample.

Figure 3.7: Difference between binding of biotin seeds in (a) the old electrode and (b)
the new electrode after electric field is turned off
Although we obtained microtubule accumulation with minus ends at the electrode tip and plus ends at the gap, the structure still doesn’t resemble a spindle since the microtubules do not overlap. To solve this problem, we set out to bind biotinylated microtubules to the electrodes and polymerize microtubule extensions off of them in the flow cell (Brown 2002). First we immobilized only biotin seeds at the electrode tip using the standard dielectrophoresis procedure. Next, we introduced rhodamine tubulin (1mg/ml in BRB80) in the presence of 4 mM MgCl₂, 1 mM GTP, 5% DMSO. After 10mins, the flow cell is washed with BRB80 to remove the background fluorescence. At room temperature, we see long microtubules that span the 20 µm electrode gap. Hence, the polymerization works to obtain microtubules that are sufficiently long to span the electrode gap. However, one issue with this approach is the skewing of microtubules in the direction of the flow (Fig 3.8). As the solutions (extension solution and wash) are introduced into the flow cell, the electrode-bound microtubules are bent in the direction of the flow (perpendicular to the long axis of the electrode) giving rise to a skewed spindle. Efforts are underway in the Hancock lab to optimize introducing solutions through a syringe pump into the flow cell. This would then facilitate to have straight spindles since the spindles would then experience less force.
Figure 3.9: Extension of biotin seeds (*red*) in the flow cell with rhodamine tubulin (*green*). Biotin seeds are immobilized on the neutravidin coated electrode using DEP. Extension solution is introduced after the field is turned off.

Thus, we have shown that assembly and dynamic growth of the mitotic spindle is possible *in vitro*. This has been achieved by the combination of AC electrokinetic techniques and protein patterning. A further development of this project would involve introducing motors like ATK5 to the artificial spindle setup and study the spindle dynamics and structure.
References:


Conclusion

The goal of this work was to build a platform that will enable us to study the interaction of single motors with the microtubules that make up the mitotic spindle. This thesis has demonstrated the feasibility of constructing artificial spindles through AC electrokinetic techniques. We have also studied *Arabidopsis thaliana* kinesin 5 (ATK5) and kinesin heavy chain (KHC) and their ability to bundle microtubules. ATK5, a minus ended plus tip tracker bundles microtubules *in vivo* at low microtubule concentrations. As microtubule concentrations are increased, most of the microtubules are recruited to form asters. We saw predominantly straight microtubule bundles. This indicates that as the motor head ambulates on the microtubule, the tail moves along maintaining straight microtubule bundles. Our results show that ATK5 motors bundle taxol-stabilized microtubules and hence the tail interaction with the microtubule is not due to plus tip localization. Overall, this is indicative of weak interaction of the motor tail with the microtubule.

We have demonstrated that microtubules can be immobilized in an antiparallel fashion to form spindles by AC electrokinetic techniques. The number of microtubules immobilized on an electrode is not affected by the microtubule being segmented. Microtubule binding is also not affected by different sample preparation processes like drying (using acetone) and neutravidin patterning (under UV conditions). However, the age of the electrode sample affects the binding, possibly due to electrode pitting or due to debris deposition on the electrode or due to electrolysis.
With the conjunction of dielectrophoresis and biotinylated microtubules interacting with neutravidin patterned electrodes, we have shown minus ends binding to the electrodes and plus ends at the gap. This is similar to the spindles in the cells which have minus ends at the spindle pole and plus ends at the center. The artificial spindles we constructed using segmented microtubules were not overlapping and hence we extended them at room temperature in the flow cell to obtain overlapping microtubules.

With this current setup of artificial spindle, it is possible to introduce motors and study their effects in spindle. Future work will involve introducing beads incubated with mitotic motors and studying the spindle dynamics made up of dynamic microtubules. ATK5 can be introduced in the spindle to study microtubule bundling and narrowing of spindle poles. Beads incubated with ATK5 can also be used to study +TIP tracking using optical traps. This can be done by trapping the ATK5 incubated beads and studying the motion of the bead. Thus, the artificial spindle setup opens up a new and useful tool to study motor proteins.