Dynamics of Microtubule Networks with Antiparallel Crosslinkers

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Dynamics of Microtubule Networks with Antiparallel Crosslinkers

A Thesis Presented

by

Kasimira T. Stanhope

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

May 2016

Molecular and Cellular Biology
Dynamics of Microtubule Networks with Antiparallel Crosslinkers

A Thesis Presented

by

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Microtubules are the most rigid element of the cytoskeleton. They are responsible for the structure of cells and make up the tracks for intracellular cargo transport. Interactions between microtubules, motor proteins, and microtubule-associated proteins drive important mechanisms in the cell, such as cell division, cell motility, cell homeostasis, and cell signaling. I seek to understand how such complex, energy-consuming non-equilibrium biological networks self-organize by studying in vitro microtubules bundled by microtubule-associated protein 65 (MAP65), in kinesin-1 gliding assays. I found that large networks can break into smaller, cell-like networks that can mimic types of cell motility. Dynamics of these networks change with varying concentrations of MAP65 and microtubules.
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CHAPTER 1

DYNAMICS OF MICROTUBULE NETWORKS WITH ANTIPARALLEL CROSSLINKERS

1.1 Introduction

Microtubules (MTs) are made up of alpha and beta tubulin dimers that form a tube of protofilaments with a cross-sectional diameter of 25 nm and can be 1-100 μm in length. They functionally have to be very ridged filaments and are known to have a persistence length of about 1 mm (Hawkins et al. 2010). The alpha and beta tubulin dimers are GTPases that always polymerize in the same direction, with one end exposing the alpha tubulin and one end exposing the beta tubulin. The end with the beta-tubulin exposed in the “plus end” that grows and shrinks faster than the alpha-exposed “minus end.” (Fig. 1)

Figure 1: Microtubules, Kinesin-1, and MAP65

A. Tubulin and Microtubule
B. MAP65
C. Kinesin-1

A. Microtubules are filaments made out of alpha and beta-tubulin subunits that form protofilaments to form a 25nm tube. Tubulin dimers are GTPases and depolymerize when hydrolyzed. 
B. MAP65 is an antiparallel microtubule crosslinker that form about a 25-35nm space between microtubules and binds every 35nm when saturated with a dissociation constant of ~1.2μM.
C. Kinesin-1 binds to microtubules and walks towards their plus ends using ATP hydrolysis. With microtubules and other MAPs they drive mitosis, cell signaling and cell transport.
Microtubules have many interactions with motor proteins. These interactions drive mitosis, cell signaling, and cell transport. Kinesin-1 is very important in active transport of signals in neurons (Goldstein and Yang 2000). This motor protein binds and walks along a single protofilament of microtubules toward the plus-end (Fig. 1) with a step-size of about 8nm (Yildiz et al 2004). Kinesin-1’s motility is fueled by ATP hydrolysis using 1 ATP molecule per step.

Cytoskeletal crosslinkers are important for microtubule organization in the cell. For example, for mitosis to occur, microtubules in the kinetochore must be aligned parallel while antiparallel microtubules are needed towards the outside of the spindle to drive the forces for mitosis. MAP65 is part of a family of crosslinkers, including PCR1/Ase1, which form antiparallel microtubule bundles (Fig. 1) that are needed for spindle formation during metaphase, telophase, and cytokinesis (Jian et al 1998). MAP65 binds to microtubules and dimerizes with other MAP65s bound to other microtubules to create a space of about 25-35 nm between antiparallel bundled microtubules (Chan et al 1999). At saturated concentrations MAP65 will regularly space itself on microtubules to dimerize about every 35 nm (Gaillard et al 2008). MAP65 is a very strong crosslinker despite having such a short residence time on a microtubule in both cells (Loïodice et al 2005) and in vitro (Subramanian et al 2010, Pringle 2013). The residence time is also shown to increase due to bundling (Bieling et al 2010).

Previous binding kinetics studies show that MAP65 has a $K_D$ value of ~1.2 μM when binding with microtubules (Tulin et al 2012). Bundling microtubules with MAP65 yields different phases of bundling resembling gas, liquid, and solid-like states as a stationary bundle (Pringle et al 2013). A previous member of my lab added microtubules pre-bundled by MAP65 to a motile flow chamber covered in kinesin-1 and observed an active state that
resembled “cell-like” microtubule organizations (Pringle et al 2013). In my thesis, I sought to expand on his observations of these “cell-like” organizations. This system is an example of an active material displaying non-equilibrium physics, which is very interesting and not sufficiently explored.

For my Master’s work, I have uncovered the state diagram of motile microtubule organization in the presence of various concentrations of MAP65 and microtubules. I performed experiments and analysis to categorize and identify dynamics of movement observed at each concentration of microtubules and MAP65. There are three phases observed by these experiments. Some bundles are extensile and are pulled completely apart by kinesin-1 motility and reach a homogeneous organization. Some bundles are contractile and create “cell-like” organization, sometimes resembling cytokinesis or a crawling amoeba. These organizations are ultimately either pulled apart, or they are released into the chamber remaining as crosslinked networks no longer in contact with the kinesin-coated surface. Other bundles contract while microtubules are pulled away from the bundle, then stop moving. My work supports previous observations that microtubules are slowed down by MAP65/PRC1 (Janson et al 2007, Subramanian et al 2010, Pringle 2013). I believe the halting of movement is due to the antiparallel bundles becoming a “knot” because it no longer has any microtubules that can be pulled free due to the bundling by MAP65. There are also bundles that do not move at all when activated. I have also explored the dynamics of bundling free microtubules while flowing MAP65 into the chamber. These experiments measure the dynamics of bundling as a function of microtubule and MAP65 concentrations.

1.2 Results and Discussion

1.2.1 Pre-Bundled Network Organization with MAP65 and Kinesin-1 Gliding
In this section of this chapter I focus on Huxley’s contribution to the evidence favoring the application of the theory of evolution to human beings. I performed in vitro reconstitution experiments combining microtubules and MAP65 at specific concentrations. In order to determine the percentage of tubulin in the microtubule with MAP65 bound, I used the hyperbolic function:

\[
K_D = \frac{[Tub]_{free}[MAP65]_{free}}{[MAP65-Tub]_{bound}}
\]  
Eq 1.

<table>
<thead>
<tr>
<th>[MT] added (μM)</th>
<th>[MAP65] added (μM)</th>
<th>% MT bound by MAP65</th>
</tr>
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<tr>
<td>1</td>
<td>0.3</td>
<td>12.87</td>
</tr>
<tr>
<td>1</td>
<td>0.98</td>
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<tr>
<td>1</td>
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<tr>
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</tr>
<tr>
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<tr>
<td>10</td>
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Table 1: % Microtubule Bound
Where \( K_D \) is the equilibrium dissociation constant, \([\text{MAP65}]\) is the concentration of free MAP65 in the solution, \([\text{Tub}]\) is the concentration of tubulin without a MAP65 bound, and \([\text{MAP65-Tub}]\) is the concentration of tubulin and MAP65 bound. From prior work, we assume \( K_D \sim 1.2 \, \mu M \) (Tulin 2011). Table 1 shows the concentrations of MAP65 we added, and the calculated percentage of MAP65 bound for each, calculated using equation 1.

Using the known percentages of MAP65 bound and how that corresponds to the phase of network formation, previously determined by the Ross Lab (Pringle 2013), I added the bundled networks of filaments to a microtubule gliding assay powered by kinesin-1 motor proteins and imaged the results using epi-fluorescence. A motility mix including ATP and an ATP regenerating system is used because it can take hours to observe a bundle come to some sort of equilibrium. First, I systematically varied the crosslinker concentration while keeping the amount of microtubules constant. Next, I held the percentage of MAP65 bound constant, while altering the microtubule density (Table 1).
1.2.1.1 Types of Activity Observed

Figure 2: Raw Data

**Extensile**: Bundle is pulled apart from kinesin-1. 3 \( \mu \text{M} \) MAP65, \( \Delta t = 15 \text{ min} \), first frame is \( t = 0 \).

**Frozen**: Bundle that is unmoving as it lands on the surface. 2 \( \mu \text{M} \) MAP65, \( \Delta t = 40 \text{ min} \), first frame is \( t = 0 \).

**Contractile**: Bundle that contracts over time. 1.5 \( \mu \text{M} \) MAP65, \( \Delta t = 20 \text{ min} \), first frame is \( t = 65 \text{ min} \).

All experiments are with 2.5 \( \mu \text{M} \) microtubules. All scale bars, 50 \( \mu \text{m} \).
Figure 2 shows example activities/patterns that I observed in mixtures of microtubules and MAP65. One common result was “extensile” activity, where bundles are being completely pulled apart by the kinesin-1 on the cover slip surface (Fig. 2A). This activity was particularly prominent at low MAP65 density. Networks of microtubules are bundled in the test tube but are pulled apart readily by the kinesin-1. There is not enough MAP65 to hold the bundles together.

At relatively high densities of MAP65 crosslinker, the networks display a frozen phase where there is no motion (Fig. 2B). In this case, I speculate that the crosslinkers are holding the bundle tightly and withstanding the kinesin-1 motility, effectively stalling the motors. We see such frozen states at the highest densities of crosslinkers.

At an intermediate regime, bundled networks contract (overall become smaller) after being added to the chamber with kinesin-1 (Fig. 2C). This is particularly interesting, as all prior work with kinesin-1 motors have shown extension. At long times (>15 minutes) these networks become pulled apart or they will either stop. Sometimes, the organizations will yield the “cell-like” organizations we saw previously (Pringle 2013).

This intermediate regime is surprising for the displayed short-term contraction and the long-term cell-like organizations. At a certain ratio of microtubules to MAP65, networks of antiparallel bundles will show a lot of pulling, knotting, and kinking in these contractile and “cell-like” phases (Fig. 2C). The networks that stop must have enough MAP65 concentrated in the center from the contraction to stall the kinesin while free MTs are still motile.

1.2.1.2 Quantitative Analysis
Although the differences described above could be detected by eye, we sought to have an automated method to identify different activities. Working with a postdoc in the lab, Dr. Vikrant Yadav, we developed a MatLab code to identify the boundary of the network for each frame and then compute statistics about the network outline (Fig. 3). For each frame, the network was outlined and the center of mass was computed. The standard deviation ($\sigma$) from the each edge point to the average center of mass was also computed. The standard deviation is a measure of the size of the network. The standard deviation was normalized compared to the initial size (standard deviation) by dividing each time frame’s
standard deviation by the initial frame’s standard deviation you get the relative standard deviation. That relative standard deviation was averaged for many networks at the same conditions (microtubule and MAP65 concentrations) and plotted versus time (Fig. 4). Error bars represent the standard error of the mean of the averages. When you plot the relative standard deviation vs. time, the trend is linear for the first 15 minutes of data. We fit a line of the form:

$$y = mx + b \quad \text{Eq. 2.}$$

using a least squares fitting algorithm to find the best fit the slope. This slope represents spreading speed of the network relative to the center of mass and edge of the original frame (Fig. 4,6).

**Figure 4: Relative Standard Dev. vs. Time**

As [MAP65] increases the relative standard deviation decreases and eventually becomes stable when frozen.
For networks that are extensile and pulled apart, the spreading speed (slope) is positive because the edge of the network is detected going further away from the average center of mass. Networks that contract display negative spreading velocities as the bundles are concentrated into the center of the network and the edge is detected getting closer to the center of mass. Frozen networks have a zero slope indicating no change in the standard deviation over time (Fig. 5,7).
Figure 6: Relative Standard Dev. vs. Time

Varied [MT]
Panel C.

As [MT] increases the relative standard deviation decreases and eventually becomes stable when frozen.
1.2.1.3 Activity as a Function of Crosslinker Density

We performed these experiments and analysis for a fixed concentration of microtubules (1 μM, 2.5 μM, 5 μM, 8 μM, and 10 μM) and systematically altered the MAP65 concentration. Figure 4 shows a data scan with 2.5 μM microtubules added and 12.89% to 56.82% MAP65 binding to microtubules. At the lowest MAP65 concentration, the spreading speed is highest (~8.5x10^-4 nm/s). As the concentration of MAP65 increases, the spreading speed decreases. Eventually, at around 34%, the spreading speed becomes negative indicating contraction of the network. At high concentrations of MAP65, the network becomes frozen with a zero slope spreading speed. (Fig. 5)
1.2.1.4 Activity as a Function of Microtubule Density

As described above, we wanted to systematically alter the microtubule density, while holding the percentage of MAP65 crosslinkers constant. In order to do that, the crosslinker concentration was also varied according to equation 1. One particular crosslinker density I studied was 12% MAP65 binding with microtubules at various concentrations. The same analysis was performed for this data and the spreading speed was determined by linear fits (Fig. 6). The determined spreading speed was plotted as a function of microtubule concentration and a similar dependence is observed with an initial extensile activity (positive spreading speed) that becomes contractile (negative spreading speed) and then frozen (Fig. 7). Interestingly, at 12% MAP65 binding, we still observed contraction when the amount of microtubules is 8 μM.

**Figure 8: State Diagram**

![State Diagram](image)

**Fig.8:** By changing the concentrations of the components we can see that there is a small regime where bundles will contract as you increase protein density before becoming frozen
Using all our data collected for a variety of concentration, we can create a state diagram of activity plotted against the microtubule concentration and MAP65 percentage of binding (Fig. 8). Interestingly, we see the low MAP65 and low microtubule concentrations are extensile and the highest concentrations of both are frozen. In the intermediate regime, we see contractile networks.

1.2.1.5 Tracer Experiments

Figure 9: Low Density Tracer Experiments

A) 99% Rhodamine Labeled

B) 1% HiLyte 647 Labeled (Tracer)

C) 1% HiLyte 647 Labeled (Tracer zoomed in)

Tracer microtubules in a different fluorescence show knotting of individual microtubules/bundles in a contractile system that later becomes frozen.
Each frame after the first has a $\Delta t$ of 500s. This is a sample of 5μM MTs and 3.1μM MAP65 (43.5% bound).

In the data above, the networks had all the microtubules labeled to show the overall network organization. I was also interested in how individual microtubules acted within the networks during these activities. In order to examine this, I performed experiments with differentially labeled microtubules (red or dark red) mixed in at a low density (1:100) with labeled microtubules of the other color (dark red or red) that made up the bulk of the network. I could track the low density microtubules as individuals while simultaneously tracking the network architecture. (Fig. 9) I observed that individual bundles and microtubules in the network appear to bend in these contractile and even extensile networks.

1.2.1.6 Control Experiments

**Figure 10: PEG Controls**

Show that PEG creates bundles that are weak enough to be readily pulled apart even at high concentrations. This is a sample of 2.5μM MTs and 5% 40 kD PEG.

I performed a control experiment bundling microtubules with poly-ethylene glycol (PEG). PEG is an inert polymer that bundles microtubules non-specifically through the depletion force (osmotic pressure). We added 5% 40 kD PEG bundles to kinesin-1 gliding assays, as above. PEG bundles are not ever as intense as the MAP65 bundles because PEG can only bundle microtubules so much until the osmotic pressure pushes PEG inside the bundles and
microtubules making them less bundled. We observed bundles that would be pulled apart readily as expected even at high concentrations. (Fig. 10)

1.2.2 Flow-In Experiments with MAP65 Bundling

**Figure 11: Flow-In Bundling**

Experiments performed to explore the dynamics of bundling free flowing microtubules to find out how long it takes to bundle and the intensity of the bundle from varying microtubule and MAP65 concentrations. All experiments were done using 2.5μM microtubules. The control and low concentrations of MAP65 show alignment from flow then become free-flowing again. Higher concentrations show super-bundling.
In order to examine the dynamics of MAP65 bundling, I performed a series of experiments bundling microtubules with MAP65 while observing in a flow chamber. These experiments are done by first imaging microtubules in a chamber with no delay, then adding a mixture of MAP65 to capture how the microtubules bundle over time. The control shows the microtubules aligning due to flow then becoming freely diffusing again over time. At low MAP65 concentrations, the same behavior is also observed. This is likely because there was not enough MAP65 to crosslink microtubules. As the concentration of MAP65 increases, the intensity of the bundles also increase (Fig. 11). This data has not yet been analyzed quantitatively, but will be done using ImageJ to compare relative intensities of bundles forming after the flow through.

1.2.3 Conclusions/Future Work

From these experiments I conclude that low crosslinker density leads to extensile activity, medium crosslinker density causes contractile activity, and high crosslinker density cause frozen, non-motile systems. The evidence of a contractile system involving kinesin-1 and MTs is rare relative to actin-myosin systems (citations here for actin-myosin), so these findings are very interesting. I am working to complete the state diagram by performing more experiments at 8 μM and 10 μM microtubules.

We are working with a theorist, Dr. Art Evans, currently a postdoc at the University of Wisconsin, to model our system. He has created simulations where he can alter the interaction strength between microtubules and the gliding speeds and friction. He is still running the simulation, but he sees similar phenomena in his simulations as we observe in experiments.
1.3 Methods

1.3.1 Making Labeled Cytoskeleton Taxol Stabilized Microtubules

Tubulin dimers are bought from the company Cytoskeleton. The lyophilized pellets are dissolved in our working buffer PEM-100 to make a concentration of 5 mg/mL. To make microtubules visible with epi-fluorescence, we combine the labeled and unlabeled tubulin to make a mixture of about 10% labeled. We centrifuge the tubulin mixture at 366,000 xg for 10 min at 4°C to pellet aggregated and misfolded tubulin from the supernatant of properly folded tubulin. Next, 1 mM GTP is added to the supernatant and incubated at 37°C for at least 20 minutes to allow for dynamic polymerizing and depolymerizing of microtubules. Next, 50 μM of Taxol is then added to the mix and incubated at 37°C for at least 20 minutes to allow for static polymerization creating long stable microtubules. We centrifuge the mixture at 16,200 xg for 10 min at room temperature to pellet the microtubules from the supernatant of inactive tubulin dimers. Finally, we resuspend the pellet of microtubules in 50 μM taxol and add PEM-100 to the original volume.

1.3.2 Kinesin-1 Purification

Transform competent BL21 E. coli cells with the pet21-GFP-Kin560-6xHis plasmid containing ampicillin resistance using LB, then plate. Create starter cultures using about 15 colonies and 5mL of terrific broth media and allow to shake at 37°C until cloudy. Cells are added to a larger culture and grown at 37°C to an O.D. at A600 of 1-2 then induced with 0.2mM IPTG. Induced cells are shaken overnight at room temperature for 16 hours.
Centrifuge induced cells to pellet the cells away from the media then allow the pellet to freeze for at least an hour. Resuspend and lyse the cell pellet in a lysis buffer (50mM pH8 sodium phosphate buffer, 250mM NaCl, 2μm/mL aprotonin, 2μm/mL leupeptin, 2μm/mL pepstatin, 0.5mM ATP, 1% Tween-20, 2mg/mL lysozyme and DnaseI) using sonication. Allow the cell lysate to incubate with silicon-nickel beads and 20.67mM imidazole at 4°C for 1.5 hours. The lysate/bead mixture was added to the column and allowed to enter completely. The column is then washed with 25mL of wash buffer (50mM pH6 sodium phosphate buffer, 250mM NaCl, 1mM MgCl₂, and 100μM ATP). Then the protein is eluted in elution buffer (50mM pH7.2 sodium phosphate buffer, 500mM imidazole, 250mM NaCl, 1mM MgCl₂, and 100μM ATP). The protein is then buffer exchanged using a NAP-5 column into our working buffer PEM-100 with 100μM ATP. Add 10% sucrose, drop freeze in liquid nitrogen and store at -80°C. The approximate concentration of the full kinesin construct is measured using BSA standards and SDS-page due to truncation.

1.3.3 MAP65 Purification

Transform competent BL21 E. coli cells with the pet28a-MAP65-6xHis plasmid containing kanamycin resistance using LB, then plate. Create starter cultures using about 15 colonies and 5mL of terrific broth media and allow to shake at 37°C until cloudy. Cells are added to a larger culture and grown at 37°C to an O.D. at A₆₀₀ of 0.6-1 then induced with 1mM IPTG. Induced cells are shaken overnight at 20°C for 16 hours. Centrifuge induced cells to pellet the cells away from the media then allow the pellet to freeze for at least an hour. Resuspend and lyse the cell pellet in 5mL lysis buffer (50mM Tris pH8, 20mM imidazole pH8, 500mM NaCl, 10% glycerol, and 1% Tween-20) per gram cell pellet using sonication five times for 30 seconds recovering on ice 5 minutes in between. Allow the cell
lysate to incubate with silicon-nickel beads and 20.67mM imidazole at 4°C for 1.5 hours. The lysate/bead mixture was added to the column and allowed to enter completely. The column is then washed with 25mL of wash buffer (50mM Tris pH8, 20mM imidazolepH8, 500mM NaCl, and 10% glycerol). Then the protein is eluted in elution buffer (50mM Tris pH8, 250mM imidazole pH8, 500mM NaCl, and 10% glycerol). The protein is then buffer exchanged using a NAP-5 column into our working buffer PEM-100 with 100μM ATP. Drop freeze in liquid nitrogen and store at -80°C. The approximate concentration of the protein is measured using a Bradford assay.

1.3.4 Pre-Bundled Gliding Assay with MAP65

I make a 10μL flow chamber using a coverglass, double-stick tape and a coverslip. I prebundle my microtubules in what will become the ATP regenerating motility mix by adding the microtubules and MAP65 to 35μM taxol, 35μM DTT and PEM-100. I allow the microtubules to incubate with the crosslinkers for 30 minutes. I flow through and fill the chamber with kinesin and incubate at room temperature for 5 minutes. During the incubation I add the remaining components to the bundled microtubules to complete the motility mix (1 mM ATP, 1.9 μM phosphocreatine, 68 μg/ml creatine phosphokinase, 4.3 mg/mL glucose, and 0.4 μl deoxy for every 10 μl, volume with PEM-100). I then flow through a BSA chamber wash (5mg/mL BSA, 60 μM taxol, 20 mM DTT, volume with PEM-100). I immediately flow through the motility mix then image using epi-fluorescence.
1.3.5 Flow in Bundling Experiments

A cross-flow chamber is made then filled with a dilution of microtubules into PEM-100 and taxol. The slide is then brought to image along with a prepared MAP65 and taxol mix. The chamber is imaged using epi-fluorescence to capture a movie of the flow-in of the MAP65 mix bundling (or not bundling) the microtubules. No incubation times are needed. Movies should be taken without delay because bundling happens quickly.
% MT bound by MAP65 Calculation:

We know that \( K_\text{D} \) is \( \sim 1.2 \mu\text{M} \)

\( K_\text{D} \) in this case is \( \frac{([\text{MT}_\text{added}]-[\text{MT-MAP}_\text{bound}])([\text{MAP}_\text{added}]-[\text{MT-MAP}_\text{bound}])}{[\text{MT-MAP}_\text{bound}]} \)

We want to find \([\text{MT-MAP}_\text{bound}]\) from \( K_\text{D} \) to calculate % MT bound by MAP65 \( \frac{[\text{MT-MAP}_\text{bound}]}{[\text{MT}_\text{added}]} \)

So we use the quadratic equation to find \([\text{MT-MAP}_\text{bound}]\) because:

\[
0 = (\text{MT-MAP}_\text{bound})^2 - (K_\text{D} + \text{MT}_\text{added} + \text{MAP}_\text{added})\text{MT-MAP}_\text{bound} + (\text{MT}_\text{added})\text{MAP}_\text{added}
\]

General Buffer:

PEM-100 Buffer: 100mM Na-PIPS, 1mM MgSO_4, 1mM EGTA, pH 6.8, store in 4°C.

Making Cytoskeleton Taxol Stabilized Microtubules: ____% labeled

Unlabeled Cytoskeleton Tubulin: The stock comes in a pellet that needs 200μL of PEM-100 added to it to be at 5mg/mL (on ice). Aliquot and drop freeze from stock. Keep sticker on tube in notebook.

Labeled Cytoskeleton Tubulin: Add 4μL PEM-100 to resuspend pellet to 5mg/mL before use. Keep sticker on tube in notebook.

Turn on large Sorvall Discovery M120 centrifuge, set to 4°C. Make sure vacuum is on.

Thaw/resuspend tubulin on ice. Transfer:

_____μL labeled + _____μL unlabeled = _____μL Total

into open round-bottom centrifuge tubes. (Make balance of same volume!)
Using the small S120AT2-0449 rotor in deli fridge, centrifuge @ 90,000rpm for 10 min.
Thaw aliquot of 100mM GTP for next step.

Discard pellet & transfer sup to a 1.5mL epp. tube. Add ____μL 100mM GTP (1mM GTP Final).

Incubate for 20 min @ 37°C.

Add ____μL 2mM Taxol (50μM Taxol Final).

Incubate for 20 min @ 37°C.

Using table-top centrifuge 5415 R, centrifuge at 13,200rpm (top speed) @ 25°C for 10 minutes. (Make a balance of same vol.!!)

Discard sup & resuspend pellet in:

____μL 2mM Taxol + ____μL PEM-100 = ____μL Total

*Use same vol. of Taxol as in step 6 and add PEM-100 to the same total vol. in step 2.

**Clean 25μL Hamilton syringe with ddH2O (fill and expel at least 3x). Use syringe to resuspend pellet (fill and expel at least 3x - try not to introduce bubbles).**

Incubate overnight @37°C (~3 nights max).

**Use syringe to declump again before use.**

**Kinesin-1 Purification**

**Day One, Transformation:**

Thaw BL21 cells on ice (if you’re also doing a miniprep thaw DH5-alpha cells too)

While cells are thawing, put agar plates in 37°C incubator

Add 5μL of plasmid to cells

Incubate on ice for 30 minutes

Heat shock @ 47°C for EXACTLY 45 seconds

IMMEDIATELY incubate on ice for 2 minutes

Add 400μL of LB

Incubate @ 37°C for 1 hour

During incubation plate 50μL of 100 mg/mL ampicillin
Once incubation is done plate 100μL of transformed cells

Incubate overnight @ 37°C

Make 500 mL Terrific Broth media in 1L flask:

- 8g Tryptone
- 6g Yeast Extract
- 1.6g NaCl
- 0.8g Na₂HPO₄
- 0.4g KH₂PO₄

Add dH₂O to bring final volume to 395.6 mL and autoclave

*4 mL 20% Glucose

*400 μL 100 mg/ml Ampicillin

*add to media after autoclaved (same day growing cells)

Make buffers if needed (sterile filter and store in 4°C):

<table>
<thead>
<tr>
<th>Lysis Buffer:</th>
<th>Wash Buffer:</th>
<th>Elution Buffer:</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM NaPO₄, pH 8.0</td>
<td>50 mM NaPO₄, pH 6.0</td>
<td>50 mM NaPO₄, pH 7.2</td>
</tr>
<tr>
<td>250 mM NaCl</td>
<td>250 mM NaCl</td>
<td>500 mM Imidazole</td>
</tr>
<tr>
<td></td>
<td>1 mM MgCl₂</td>
<td>250 mM NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mM MgCl₂</td>
</tr>
</tbody>
</table>

**Day Two, Induction:**

1. First thing in the morning, make 5 mL starter culture:

- 5 mL TPM media
- 50 μL 20% Glucose
- 5 μL 100mg/ml Ampicillin
- 15 colonies from transformation plate
2. Incubate starter culture on 37°C shaker until culture is cloudy (approximately 2 hours)

3. Once starter culture is cloudy, add to 400 mL TPM media (*add glucose and ampicillin to TPM media). Set aside 2 ml of media without bacteria for control on the OD600.

4. Grow cells to OD 1-2 (ideal 1.7) in TPM media. Monitor the growth using OD600.

5. Let culture cool to room temperature (save 20ul for pre-induced sample)

6. Induce with IPTG (final concentration of 0.2 mM)

7. Shake at room temp overnight.

**Day Three, Protein Purification:**

1. **Save 20 μl induced cells for gel sample (Induced Cells).**
   
   Spin down cells for 10 minutes at 4°C, 5,000 rpm

2. Pour off supernatant, freeze pellet in -80°C for 1 hour
   
   **pellet can also be left at -80°C for purification on a different day**

3. Put pellet on ice and resuspend in 30mL cold Lysis Buffer (keep on ice during resuspension)
   
   To Lysis buffer, add: 30 μl 2mg/mL Aprotonin
   30 μl 2mg/mL Leupeptin
   30 μl 2mg/mL Pepstatin
   30 μl Tween-20
   150 μl 100mM ATP

4. Add 62mg Lysozyme

5. Rock at 4°C for 20 minutes

6. Add 15ul DnaseI (Sigma)

7. Sonicate 1 minute on ice (setting 3)

8. Centrifuge 30 minutes, 4°C, 40,000xg (T865 rotor)

9. Save a small sample of the pellet (dark brown) as gel sample. Save 20 μl of the supernatant as gel sample.
Bead Equilibration: (if you have a Ni column, this is not needed)

*put beads on rocker for ~30 minutes to get beads fully into solution before using

1. Add 500ul Ni beads to 2mL tube, add 1.5mL Lysis Buffer, invert tube 10 times, spin 2 min at 500xg, remove sup without disturbing bead bed
2. Repeat for a total of 4 times
3. Pour supernatant into 50mL conical tube Take a 20 μL gel sample.
4. Add 620 μl 1M Imidazole to supernatant
5. Add some supernatant to beads, and bring back and forth to get beads into supernatant conical
6. Incubate at 4°C on rocker for 1.5 hours

Buffers with ATP:

<table>
<thead>
<tr>
<th>Wash Buffer:</th>
<th>Elution Buffer:</th>
<th>PEM-100:</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mL Wash Buffer</td>
<td>2.5 mL Elution Buffer</td>
<td>12 mL PEM-100</td>
</tr>
<tr>
<td>25 μL 100mM ATP</td>
<td>2.5 μL 100mM ATP</td>
<td>12 μL 100 mM ATP</td>
</tr>
</tbody>
</table>

1. Flow Lysate sup+beads through column (*gel sample of FT*)
   *Lysate FT may be put in -80°C freezer and used for another purification
2. Flow 25mL Wash Buffer (with ATP added) over column (*gel sample of FT*)
3. Flow 150 μL Elution Buffer+ATP over column and collect first fraction (*gel sample*)
4. Flow 500 μL Elution Buffer+ATP over column for fractions 2-5 (*gel samples*)
   *kinesin will usually be in fraction 2 (sometimes I make all fractions 150uL to make it more concentrated)
5. dot blot to verify fraction containing kinesin:
   -with pencil, draw 5 circles on a piece of filter paper, label circles Elutions 1-5
- add 0.5 μL of each fraction to respective circles
- stain with coomassie for 30 seconds
- destain, observe the glory

**Buffer Exchange:**

1. Remove top and bottom cap of NAP-5 column
2. Allow buffer to completely drain by gravity flow
3. Add 10 mL PEM-100+ATP and allow to completely enter gel bed
4. Add 500 μL of elution with protein (determined by dot blot)
5. Put new 1.5 mL tube under column
6. Add 1 mL PEM-100+ATP and collect eluate (you may want to break this up into multiple small volumes and do another dot blot to determine which fractions to use to make more concentrated) (*20 μL gel sample*)
7. Add 200 μL 60% sucrose (10% final concentration) to buffer exchanged kinesin
8. Aliquot
9. Drop freeze in liquid nitrogen and store in -80°C

**MAP65 Purification**

Buffers:

Terrific Broth Media: 6g tryptone, 12g yeast, 2mL 100% glycerol, and volume to 450mL w/ddH₂O

Potassium Phosphate Buffer: 1.16g KH₂PO₄, 6.25g K₂HPO₄, volume with ddH₂O to 50mL, pH~7

Lysis Buffer: 50mM Tris pH8, 20mM imidazole pH8, 500mM NaCl, 10% glycerol, and 1% Tween-20

Wash Buffer: 50mM Tris pH8, 20mM imidazolepH8, 500mM NaCl, and 10% glycerol

Elution Buffer: 50mM Tris pH8, 250mM imidazole pH8, 500mM NaCl, and 10% glycerol

Day 1, Transformation
Transform E. coli BL21(DE3) or Rosetta(DE3) cells with pET28a-based expression vector with your favorite gene. We typically use pET28a-TEV (pTEV), which has the TEV cleavage site.

Plate cells on LB-Kanamycin (50 mg/l) and grow overnight at 37 °C.

Day 2, Inoculation/Induction

First thing in the morning, inoculate 5 ml LB-Kan culture with 15 colonies.

Grow at 37°C, 250 rpm until cloudy (approx. 2 hours).

Once starter culture is cloudy, inoculate 500ml Terrific Broth + Kanamycin (50 mg/l) with the 5 ml culture and grow to a cell density of $A_{600}$ of ~ 0.6-1.0. Set aside 2 ml of media without bacteria for control on the OD600.

Monitor cell culture OD every 30-60 min.

Save 10 μl pre-induced cells for gel sample.

Induce protein expression by adding 1 M sterile IPTG to a final concentration of 1 mM IPTG.

Shake overnight hours at 20°C, 250 rpm. Low temperature induction is essential to prevent protein aggregation and exclusion into inclusion bodies.

Save 10 μl induced cells for gel sample (Induced Cells).

Day 3, Purification

Save 10 μl induced cells for gel sample (Induced Cells). Harvest cells by centrifugation at 5000 xg, 10 min, RT in GSA bottles. Weigh the cell pellet (typically 3-4 grams per 500 ml). Freeze the pellet at -20°C until ready to do protein purification, if needed.

Thaw the cell pellet at 37°C for 1-2 min and then keep on ice for 5 min.

From this point on, keep cells/lysate on ice at all times.

Resuspend cells in 5 ml ice-cold lysis buffer per gram cell pellet. Stir the pellet to resuspend slowly at 4°C. Avoid frothing!!

Transfer cell suspension to a glass beaker and sonicate on ice for a total of 5 times, 60% duty, power setting 6, for 30 s each time. Let the cells recover on ice for 5 min between each cycle. Be careful to place the sonicator probe so that the tip is immersed in the center of the resuspended pellet and NOT touching the side of the beaker.

At the end of sonication the lysate looks translucent.
Centrifuge cell debris at 10,000 xg for 30 min 4 °C in SS34 rotor.

Do this while the centrifugation is occurring: Add 500 μl Ni beads to 2 ml tube, add 1.5 ml Lysis Buffer, invert tube 10 times, spin 2 minutes at 500 xg, remove supernatent without disturbing bead bed.

Repeat for a total of 4 times.

Transfer supernatant into fresh cold 50 ml tube immediately. Avoid getting any pellet in the supernatant as this will clog the Ni column. Better to collect only 90-95% of the supernatant to avoid getting any pellet contamination.

Save a small sample of the pellet (dark brown) as gel sample. Save 50 μl of the supernatant as gel sample.

Use supernatant for Ni-bead purification.

Pour the entire supernatant fraction into a 50 ml conical tube. Take a 20 μl gel sample.

Add 620 μl 1M Imidazole to supernatant.

Add some supernatant to beads, and bring back and forth to get beads into supernatant conical

Incubate at 4°C on rocker for 1.5 hours at 4°C.

Flow the supernatant with beads into the column. Catch the flow through in a 50 ml conical tube labeled “Flow Through” and save a 20 μl gel sample. Save the Lysate Flow Through in the -80°C for future purification.

Wash column once with 25 ml ice-cold Wash Buffer to remove unbound proteins. Save sample for gel and stopper the column before the bed resin gets dry.

To elute protein, prepare 6 eppendorf tubes labeled with fraction numbers (1-6).

While the column is still stoppered, add 3 bed volumes of Elution Buffer and immediately unstopper the column and collect fractions.

Fraction 1: 0.5 ml (mostly junk)

Fraction 2: ~ 1 ml (peak protein fraction)

Fractions 3-6: ~ 1 ml each.

Check for protein elution by performing a dot blot:

With pencil, draw 5 circles on a piece of filter paper, label circles Elutions 1-5

Add 1 μl of each fraction to respective circles
Stain with Coomassie for 30 seconds

Destain until protein dots are clear and darker than the background.

Save 20 μl gel samples of each fraction.

Desalt using PD-10 column or similar into PEM-100.

Remove top and bottom cap of column.

Allow buffer to completely drain by gravity flow.

Add 10 ml PEM-100+ATP and allow to completely enter gel bed.

Add 500 μl of elution with protein (determined by dot blot).

Put new 1.5 ml tube under column.

Add 1 ml PEM-100+ATP and collect eluate. Save 20 μl gel sample.

Aliquot into 20 μl aliquots.

Drop freeze in liquid nitrogen and store in -80°C.

Pre-Bundled Gliding Assay with MAP65

For the gliding assays using pre-bundled microtubules, the following Motility Mix was required:

Pre-bundled MT, ATP Regenerating, Motility Mix: 35 μM Taxol, 35 mM DTT, desired amount of MAP65 and microtubules (see below for the pre-bundled microtubules), 1 mM ATP, 1.9 μM Phosphocreatine, 68 μg/ml Creatine Phosphokinase, 4.3 mg/mL glucose, and 0.4 μl deoxy for every 10 μl, volume with PEM-100. Add the ATP, Phosphocreatine, Creatine Phosphokinase, glucose, and deoxy just before insertion into the chamber.

Deoxy: 10 mg/mL glucose oxidase, 4.7 mg/mL catalase, in double distilled water. Spin down solids and remove the saturated solution at the top. Store in 4°C for up to a week.
BSA Wash Buffer: 5mg/mL BSA, 60 μM Taxol, 20 mM DTT, volume with PEM-100. Make the
day of the assay and do not store for reuse.

**Experiment:**

In order to obtain dynamic organizations of microtubules that continue to be motile in the
chamber, we need to pre-bundle microtubules by mixing microtubules with MAP65.

Make Pre-bundled MT, ATP Regenerating, Motility Mix and incubate for at least 30 minutes
to bundle microtubules. Do not include the ATP, ATP regenerating system, or deoxy.

Make Chamber Wash and standard flow chamber (13.1.1).

About 10 minutes before make Pre-bundled MT, ATP Regenerating, Motility Mix incubation
is done take out kinesin to thaw in hand.

About 5 minutes before incubation is complete, flow in 10 μl of kinesin. Incubate for at least
5 minutes. Another aliquot may be needed depending on number of active kinesin in the
preparation, and should be assessed with the standard gliding assay.

When incubation is complete add ATP, glucose, deoxy, and regenerating system.

Flow in 10 μl of chamber wash to the flow chamber.

Flow in 10 μl of the Pre-bundled MT, ATP Regenerating, Motility Mix and image
microtubules using epi-fluorescence with shuttering between frames. If bundles are
particularly three-dimensional, time series imaging can include z-stacks using a motorized
stage with a z-stepper.

**Flow-in Dynamic Bundling**

Reagents:

PEM-100: 100mM Na-PIPES, 1mM MgSO₄, 1mM EGTA, pH 6.8, volume with double distilled
water

Microtubule Dilution: 1μL 2mM Taxol, 1μL stock microtubules, volume to needed
concentration with PEM-100
MAP solution: 1μL 2mM Taxol, amount of MAP needed, volume with PEM-100 to needed concentration

Make a standard cross-flow chamber

Flow in 10μL of microtubule dilution and image using epi-fluorescence with no delay towards one end of the chamber.

On the other end of the chamber flow in 10μL of MAP solution and observe bundling.
LITERATURE CITED

Bieling P, Telley I A and Surrey T 2010 A minimal midzone protein module controls formation and length of antiparallel microtubule overlaps Cell 142 420-32


