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Chapter 2

Building the gliding motility assay

2.1 Introduction

A great number of tools are used in gliding motility assays. These tools come in the form of chemicals, proteins, equipment, and procedures. As with any biological experiment, there are numerous variables and any number of those variables can cause problems in the experiment. By understanding the chemicals and proteins used and observing my previous failures, I can now pinpoint where a problem exists that caused the assay to go awry. Thankfully I have been able to collect enough data to show how this assay can go awry and, I hope that my failures can be of some use to others who do these types of experiments.

In this chapter, I will discuss the various procedures I use to make a gliding motilty assay. In the following chapters, I will discuss how changes to the assay are made in order to probe different aspects of the kinesin and microtubule system.

2.2 Methods and materials

There are several chemicals used in the gliding motility assay. The fun part of these experiments are when changes to the chemicals are made to try and fish out some of

the physics about the interactions of kinesin and microtubules. Before I talk about changing chemicals, I will describe my procedure on how to make the basic buffer solution used for nearly all experiments; the PEM buffer. I will then go into how to make the other buffers and solutions necessary to run a basic gliding motility assay.

2.2.1 The PEM buffer

PEM buffer overview

PEM, also known as BRB80, is the basic buffer used in all the gliding motility assays. It was found that tubulin polymerized quite nicely in this buffer with maximum effectiveness occurring at similar concentrations to what we use today. Olmsted and Borisy [Olmsted 1975] showed that microtubules polymerized very well in a solution that contained approximately 100 mM PIPES, 1 mM EGTA, and stoichiometric ratios of $MgCl_2$ to tubulin. Since microtubules polymerize so well in this buffer, it seems only natural to keep it in everything. This is why PEM is used in this assay and why I make sure to put all my proteins in this buffer.

Before I discuss the recipe of PEM that I have used for nearly all my experiments, I will first discuss the ingredients to the buffer. I will also discuss the chemicals, how I store them, and link to the exact products used in the subsequent experiments. PEM stands for:

- **P**IPES
- **E**GTA
- **M**gCl_2

I have adopted this terminology as a mnemonic and, it goes well with the naming convention of the other solutions necessary for the experiment. One of the difficulties with working with many different chemical solutions is agreeing on a common

language and a naming convention. Unfortunately there is no common naming convention in the literature and as such, it can be difficult for a beginning researcher in this field to understand what buffers one group or another uses. In fact, when I was first attempting to learn the assay, I was presented with a buffer solution called BRB80 that had no explanation as to why it was called this. Apparently, no one else knows what the initials stand for either, as the link would suggest. I hope that the naming convention I have proposed is straight forward and easy to use for other researchers.

PIPES is an acid and is the pH buffer used in PEM. It has a pK_a of 6.76 which is why PEM is pH-ed very close to this value. Unfortunately, there is no consensus in the literature about which form of PIPES to use in PEM, i.e. K_2PIPES or Na_2PIPES. Neither is there a consensus on whether to use sodium or potassium in solution or why one may be better than the other. See for instance Ray etal. [Ray 1993] for an example of a buffer pH-ed with KOH and Woehlke et al. [Woehlke 1997] for an example using NaOH. After discussing this with my P. I., Dr. Koch, we chose to use the acid form (no K^+ or Na^+ attached to it) of PIPES. Since this form of PIPES did not have any sodium or potassium ions on it, we could actively choose which counter ion we wanted in solution. I decided to use NaOH in my version of PEM which means I probably should call it Na-PEM but, I've dropped the Na for simplicity. If I were to use KOH, then I would probably call it K-PEM. The stock chemical of PIPES is stored at room temperature in the desiccator in its original bottle.

EGTA chelates both calcium and magnesium from solution and is also an acid. EGTA has a higher affinity for calcium than it does for magnesium which is good since for motility to work, magnesium must be in solution [Olmsted 1975]. I have yet to find an article that describes why EGTA is used in PEM. I believe that EGTA is used in PEM because I think it will chelate calcium phosphate found in casein micelles. Casein is used to passivate glass and is discussed in detail below. The chelation of

calcium phosphate breaks apart casein micelles and I believe that breaking up the micelles aids in surface passivation. In their book, Fox and McSweeney ([Fox 1998a] state that EDTA disintegrates casein micelles, however, they do not discuss EGTA disintegrating casein micelles. EDTA and EGTA are very similar compounds as both are magnesium and calcium chelators. Holt et al. [Holt 1994] talks about the similarities of EDTA and EGTA which were used to investigate the ratio of calcium to phosphate in milk. These two references make me inclined to believe that EGTA will disintegrate casein micelles similarly to how EDTA does it. In fact, I believe that EGTA may do a better job of breaking up the micelles since EGTA has a higher affinity for calcium than EDTA does. EGTA is stored in the desiccator at room temperature in its original bottle.

Magnesium is essential for both the polymerization of microtubules [Olmsted 1975] and for the motility of kinesin [Böhm 1997]. This is why magnesium chloride is included in the PEM buffer. $MgCl_2$ is purchased in solution at a concentration of 1 M in water. I do not purchase the salt form of $MgCl_2$ because it is extraordinarily hygroscopic. So much so that if you leave a pellet of it out, it will suck up so much water from the atmosphere that it will basically put itself into solution. Even in the dry New Mexico air. Since $MgCl_2$ is in solution already, it does not need to be stored in the desiccator and it can be stored at room temperature in its original bottle.

Since the pH buffer PIPES and the divalent cation chelator EGTA are acid forms of the chemicals, counter ions must be in solution in order for them to dissolve. As previously discussed, those ions come in the flavor of NaOH.

Getting the correct amount of NaOH for a solution of PEM was tricky at first, however, now I know the approximate amount to use because of trial and error. Scientists typically will not state how much of a pH-ing chemical is added to a buffer. I'm not sure why this is the case. Especially since it is known that changing the ionic strength of the motility assay does affect gliding speeds [Böhm 2000a]. NaOH comes

in pellet form and should always be dessicated due to its hygroscopic nature. It is also a very strong base so care must be taken when handling it. When using this chemical one must work quickly, otherwise it will pull water from the atmosphere and throw off weight measurements. NaOH should be stored at room temperature in the desiccator at all times.

Ultimately these chemicals, PIPES, EGTA, $MgCl_2$, and NaOH must be put into an aqueous solution. We have a reverse osmosis deionizing water system that produces very pure 18.2 MΩ-cm H_2O. Water is very important in the experiments that I conduct. I will discuss in greater detail some properties of water and its isotopes in Chapter 3.

PEM buffer recipe & procedure

This PEM buffer is not unique and neither is it the standard buffer used for gliding motility assays. See here for a few other labs' "PEM" buffers.

I prepare a 10x concentrated version of the PEM buffer used in assays. I do this for two reasons; the first is when I make TSB which is discussed below and second is because 10x PEM is used in another assay that contains $H_2{}^{18}O$ water. Also, I can make a smaller volume of 10x PEM and store it more easily than I can a 1x PEM solution. The 10x PEM buffer contains the following concentrations of chemicals.

- 800 mM PIPES
- 10 mM EGTA
- 10 mM $MgCl_2$
- ≈ 1.25 M NaOH
- 18.2 MΩ-cm H_2O

The procedure I use to make this buffer is as follows:

1. Weigh out the appropriate amount of PIPES, EGTA, and NaOH to make a 25 mL solution. This comes out to:

    - 6.0474 g PIPES
    - 0.0951 g EGTA
    - 250 μL $MgCl_2$
    - $\approx$ 1.2 g NaOH — I like to keep the amount of NaOH below the 1.25 M upper limit. That way I don't over shoot the amount of NaOH needed. If I do end up going past the pH goal of 6.89, I will restart this procedure since I do not want to have HCl in the buffer.
    - All components are placed in a 50 mL centrifuge tube and vortexed with just enough 18.2 MΩ-cm H_2O such that the chemicals will completely dissolve. I never put more than 15 mL of water in the tube at this step.

2. Once all the chemicals are in solution, I add more 18.2 MΩ-cm H_2O to the tube till the total volume is about 22 mL. I don't add the total amount of volume needed (25 mL) since I know that I may have to pH the buffer. Having less than the total volume needed ensures that I don't add too much water to the solution thus diluting the chemicals if I need to pH it.

3. Prepare a solution of 10 N NaOH, typically about 10 mL worth in 18.2 MΩ-cm H_2O just in case pH-ing is needed.

4. Determine the pH of the solution. Add small amounts of the 10 N NaOH prepared in the previous step if needed in order to reach the 6.89 pH. If I over shoot this value, I scrap the buffer and start again.

5. Once the correct pH is achieved, add the appropriate amount of water to reach the 25 mL total volume mark.

6. The PEM solution is then syringe filtered using a 0.2 μm filter and aliquoted into 1 mL screw top vials that are then labeled and stored in the 4°C fridge in a convenient fridge box.

2.2.2 Surface passivation chemicals

Casein overview

In the gliding motility assay, motility is sustained by first passivating the glass microscope slides. Passivation is done to prevent kinesin's motor domains from becoming inactive when interacting with untreated glass. It is not understood how or why kinesin motor domains become inactive on untreated glass but they do. Passivation of glass can be done with bovine serum albumin (BSA) ([Böhm 1997], [Böhm 2000a], [Böhm 2000b]) bovine casein ([Ozeki 2009], [Woehlke 1997], [Moorjani 2003], [Hess 2001], [Ray 1993]), a lot of kinesin [Verma 2008], or other chemical compositions [Howard 1989]. Bovine casein is the surface blocker of choice by many experimenters mainly because it works well at passivation and is inexpensive. 500 g of whole bovine casein costs about $30 at the time of this writing. Typical assays will use 10 — 50 μg of casein at a time. This means that the 500 g stock of bovine casein will outlast a graduate student's career and if stored properly, possibly a PI's.

Casein is a globular protein that does not have a known crystal structure [Verma 2008], see Figure 2.1. Bovine casein is comprised of four major subgroups: α_{s1}, α_{s2}, β, and κ. Depending on the mammal the caseins come from, there exists different ratios of these globular constituents. For instance, bovine casein contains $\alpha_{s1} + \alpha_{s2} > \beta > \kappa$ and human casein contains $\beta > \kappa$ with only trace amounts of α_{s1} casein ([Fiat 1989], [Fox 1998a]). Each species has a finely tuned milk for their neonates as casein is a vehicle in milk for delivering calcium phosphate and amino acids to them.

Figure 2.1 also shows a theory about how the casein micelle looks, for a discussion of the other theories, see the review done by Phadungath [Phadungath 2005]. Since whole casein can not be crystallized, no one really knows for sure what the micelle looks like inside. What this model suggests is that kappa casein is more than likely on the outside of the micelle and is there to stabilize its overall size. Alpha and beta caseins form complexes that are inside the micelle and help to sequester calcium phosphate.

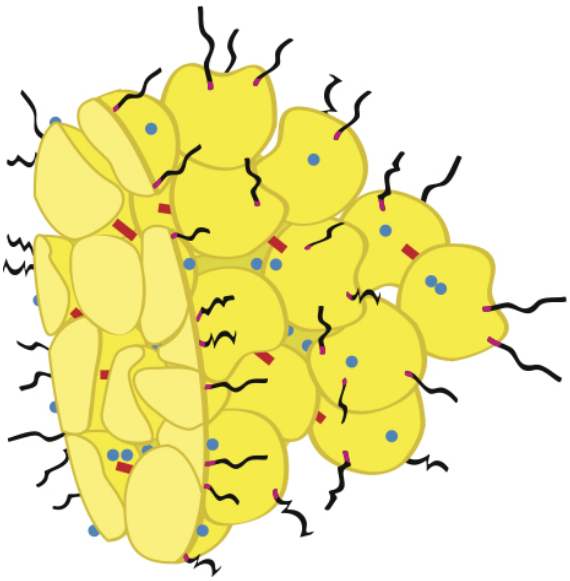

Figure 2.1: Graphical representation of a bovine casein micelle made by Anthony Salvagno. Since whole casein is not crystallizable, this image may or may not be accurate but it is one theory about how whole casein looks. Alpha and beta casein form a globular "sub-micelle" complex (yellow balls) that are stabilized by kappa casein (black lines). The casein sequesters calcium phosphate from solution (red and blue markers).

How casein passivates glass surfaces in order to support kinesin for the gliding motility assay is still not very well understood but, some work has been done to try and understand it. Ozeki et al. showed that two layers of casein form on the glass surface to help support kinesin for motility [Ozeki 2009]. Verma et al. [Verma 2008] showed that the number of microtubules that landed on the kinesin surface was affected by the casein passivation. Hancock and Howard also showed that the number of microtubules that landed on the kinesin surface was dependent on the number of motor proteins adhered to the glass slide [Hancock 1998]. A visual aid of how we think passivation occurs can be found in Figure 2.2. Still, no one knows if kinesin adheres to the top layer of the casein passivation or, if it embeds itself into the passivation layer.

With all these studies showing how the kinesin and microtubule system can be

(Passivation movie.)

Figure 2.2: Click here to view the movie via YouTube. Click the movie to play, press P to pause. Movie showing how passivation occurs and how a gliding motility assay works. The orange globs are casein micelles, the blue and green items are kinesin and the large green rod like object is a microtubule. No one knows if kinesin adheres to the casein passivation or, if it embeds itself into the passivation layer.

affected by the passivation substrate, one can assume that the system will be affected by the individual components of bovine casein. I will discuss this line of thinking more in detail in Chapter 2. For now, I will outline how to prepare a basic solution of whole casein in PEM which is what most researchers use as the passivation chemical for conducting gliding motility assays.

Whole casein does not dissolve very easily without the addition of heat. Thankfully, whole casein does not have a secondary structure and thus does not denature when heated [Fox 1998b]. This is why milk can be subjected to ultra high temperature (UHT) pasteurization with minimal ill effects. I have never tried low temperature pasteurized milk but I hear that the milk tastes better. UHT heats milk above 135°C for very short periods of time in order to kill bacteria. Low temperature pasteurization uses much lower temperatures for much longer periods of time to obtain the same results.

The type of whole casein I use in experiments comes from Sigma. I should point out that I also tried a Vitamin free version of whole casein as a passivator. I could not get this casein to dissolve in PEM very easily and thus I decided to use the "technical

grade" whole casein from the link above.

W-PEM recipe

When I prepare whole casein solutions, I use a condenser since heating up the solution will cause evaporation and the use of a condenser eliminates that problem, see Figure 2.3. If I didn't use a condenser, then I would have to replace the water that evaporated back into the flask. This is cumbersome since one never really knows for certain how much of the water evaporated.

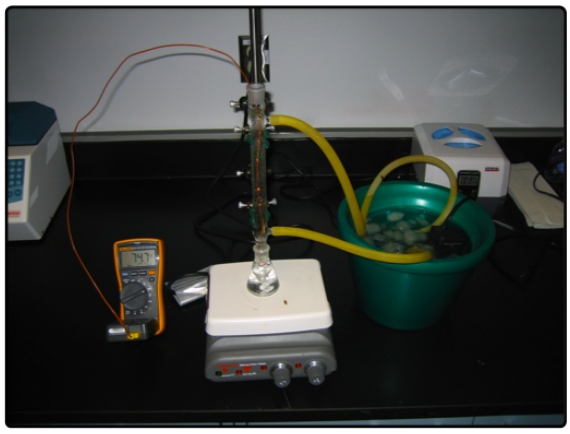

Figure 2.3: Condenser unit attached to a solution of whole casein being mixed and heated in PEM.

I will prepare whole casein at a concentration 1.0 mg/mL in PEM. I call this solution W-PEM to differentiate it from the other solutions of casein that are prepared for other studies discussed in Chapter 2. To prepare W-PEM, I do the following.

1. Setup the condenser, stir/hot plate and a temperature probe.

2. I typically make 25 mL of W-PEM so I weigh out 25 mg of whole casein.

3. I add the whole casein to the flask and then 25 mL of PEM. PEM is made from the 10x concentrated PEM stock solution by dilution.

4. Stir the solution at the highest setting on the stir plate. If my stir/hot plated allowed me to take the stirring up to 11, I would have.

5. Turn on the hot plate to a medium low setting, 3 for my case.

6. Monitor the temperature of the solution with a temperature probe. Once it reaches between 60 — 80°C, turn off the heat and continue to stir. The heating

time is about 15 minutes for my setup. If there is still casein in solution after the 15 minutes, I will keep the hot plate on till I no longer see visible precipitates of whole casein.

7. Let the hot plate cool back to room temperature while still stirring the W-PEM.

8. Once the hot plate is cool, I will remove the flask from the condenser and cover it with Parafilm. There should be a foam on top of the solution. This foam indicates that the casein has been dissolved.

9. I will then put the W-PEM in the 4°C fridge over night to ensure that the foam has incorporated itself back into solution.

10. After the foam has disappeared, I will aliquot the W-PEM into convenient screw top vials and store them at 4°C.

If the casein solutions are kept at 4°C, they will last for up to 6 months. I do not filter this solution. The reason why I do not filter it is because I do not want to loose any of the casein in the filter. Not filtering it ensures that I have a 1.0 mg/mL solution of whole casein in PEM.

2.2.3 Antifade

Antifade overview

I use a very common antifade system that consists of the following components.

- Glucose Oxidase (GOD).
- Catalase (CAT)
- β-mercaptoethanol (BME).
- D-glucose.

The antifade system is vital for observing a good gliding motility assay and is used because it prolongs the observation time of fluorescent microtubules. Since I observe my microtubules with fluorescence, elongating the time it takes before the microtubule fades is crucial for taking good data with a high signal to noise ratio. I rather dislike using this antifade system because the BME has a very strong sulfur odor. There are other recipes for antifade systems that exist [Aitken 2008], however, I have not preformed any experiments with them.

Glucose oxidase requires D-glucose in solution as this is its fuel source. GOD oxidizes D-glucose to gluconic acid while using up oxygen in the solution. This is a good thing since photobleaching is caused from highly reactive oxygen species. Unfortunately when GOD oxidizes D-glucose, it also produces hydrogen peroxide which can damage the kinesin microtubule system. Catalase is added to the mix in order to decompose the hydrogen peroxide. BME, β-mercaptoethanol, or 2-mercaptoethanol is used to prevent blinking of the fluorophore and to quench triplet states ([Aitken 2008], [Rasnik 2006]). Glucose oxidase and catalase should be stored in the -20°C freezer and BME can be stored in the 4°C fridge all in their original containers.

BME is nasty stuff and smells quite horribly. If any of this stuff spills anywhere, the stench will permeate through the lab for days. I know this because it has happened in my lab. Even a microliter will stink up the place very badly. Anything that touches BME needs to be handled carefully and disposed of, or cleaned properly.

Antifade components

The antifade chemicals are mixed in PEM and stored in the -80°C freezer. Below I outline the stock solutions necessary to prepare the antifade cocktail. In the recipe section, I describe the recipe that I use to prepare what I call antifade.

PEM-GOD Weighing out the amount of glucose oxidase needed to prepare a stock solution of PEM-GOD can be difficult since such a small quantity is needed. I typically opt to make a 1000x more concentrated solution than what is needed in the motility assay since

it is much easier to weigh out at this concentration. I will then dilute the 1000x solution to a 100x solution as this is what is needed for the antifade cocktail. 1000x PEM-GOD contains:

- 20 mg/mL Glucose oxidase in PEM

I only make 1 mL of PEM-GOD at a time. This solution will keep for 6 months in the -80°C freezer in a screw top vial.

PEM-CAT Again, weighing the amount of catalase needed is difficult. I opt for making a 1000x concentrated solution of CAT in PEM and then dilute it by a factor of 10 to obtain the 100x solution needed for the cocktail recipe. 1000x PEM-CAT contains:

- 8 mg/mL Catalase in PEM

I only make 1 mL of PEM-GOD at a time. This solution will keep for 6 months in the -80°C freezer in a screw top vial.

BME BME does not have to be diluted in PEM. It is used as is from the stock solution purchased from Sigma.

PEM-Glu Along with the above two solutions, I make up what I call PEM-Glu. PEM-Glu is nothing more than a 2M solution of D-glucose in PEM. Yes. I want to make a 2 M solution of glucose, which means that there is a lot of sugar to put into a very small volume of liquid. This is an extreme case where the volume of the solute has to be taken into consideration. If I measure out the total volume of liquid I want to make a 2 M solution of glucose with and just add the glucose to it, I will end up with a solution that is a larger volume than I anticipated. To prevent this from happening, I weigh out the glucose and add it to a smaller volume of liquid than my final target total volume. I vortex it so that a considerable portion of the glucose goes into solution and then add more PEM till I reach my target volume. I only make 1 mL total volume of PEM-Glu and aliquot it into 20 μL aliquots and store them in the -80°C freezer. D-glucose should be stored at room

temperature in its original container. I should note that the PEM-Glu is not added to the antifade system aliquots, it's added to the motility solution before observations.

Antifade recipe

The recipe and procedure for this antifade system is as follows.

- More importantly than the exact volumes listed below is the ratio of the chemicals used, i.e. PEM-GOD:PEM-CAT:BME is 2:2:1.

    1. 12 μL of 100x GOD is added to a microcentrifuge tube.
    2. 12 μL of 100x CAT is added to the same microcentrifuge tube.
    3. In the hood with the fan on, 6 μL of BME is added.
    4. Vortex the solution and spin it down.
    5. Aliquot the antifade into 5 μL aliquots stored in microcentrifuge tubes.

Antifade solutions stay viable for only one week if stored in the -20°C freezer.

2.2.4 Tubulin

Here I will describe how to store tubulin and the various solutions necessary for microtubule polymerization. For a brief description of what tubulin and microtubules are, please refer to the Introduction. The tubulin used in the lab comes from bovine brains and is purchased from Cytoskeleton. Thankfully, there is a company that sells purified tubulin. This is great since I'm not too keen on having to harvest tubulin myself, which involves liquefying cow brains [Shelanski 1973].

Tubulin from Cytoskeleton arrives lyophilized (flash frozen) in 1 mg, 20 μg, and 20 μg aliquots respectively. Aliquots are stored in the -80°C freezer at all times upon arrival. The gliding motility assays use rhodamine labeled tubulin exclusively, as it gives a much better signal to noise ratio than does the fluorescein labeled tubulin.

Polymerization of tubulin is performed in a tubulin suspension buffer. As mentioned above, one can change this buffer to polymerize microtubules with varying numbers of protofilaments.

Tubulin Suspension Buffer (TSB) overview

The tubulin suspension buffer (TSB) used in the lab contains the following components.

- 1.06x PEM
- $MgCl_2$
- GTP
- 6% (v/v) Glycerol

The usage of 1.06x PEM is necessary since I add glycerol by volume to the TSB. If I did not use 1.06x PEM, then the addition of glycerol to TSB would dilute the PEM buffer.

The lab has chosen to add an extra 1 mM $MgCl_2$ in the TSB since EGTA chelates Mg^{2+} from solution. In order to ensure that polymerization occurs efficiently, we decided to add an extra 1 mM $MgCl_2$ to TSB in order to counteract the chelation of $MgCl_2$ by EGTA.

GTP is necessary for efficient microtubule polymerization. It is highly toxic so care must be taken when using this chemical. Preparing this suspension in the hood is highly recommended in order to prevent any inadvertent inhalation of GTP dust, as it definitely burns a lot if inhaled.

Glycerol is extraordinarily viscous and is a terrible pain to try and measure out. I measure it with our 1000 μL Eppendorf pipettor. The 1000 μL pipettor produces enough force to overcome glycerol's viscosity while the 100 μL pipettor unfortunately does not. This puts a lower bound on the amount of TSB that I need to make, i.e at least 1666.7 μL since the lowest setting on the 1000 μL pipettor is 100 μL. Getting all the glycerol needed in the pipettor takes some Zen motivation as it requires patience to wait until the proper amount of glycerol has been measured out. As Heinz ketchup would say back in the 1980's "Good things come to those who wait" and it does take a while to measure the proper amount of glycerol.

Glycerol is used to speed up microtubule polymerization [Keates 1980]. Other chemicals can be used in polymerization from DMSO to excess Taxol. These three polymerization techniques result in three different types of microtubules being polymerized with the major difference in the microtubules being the number of protofilaments [Ray 1993].

TSB components

1.06x PEM This is just a dilution of 10x PEM in water. I only make the amount necessary to make a batch of TSB and to suspend the GTP in solution. To make a total amount of 5 mL 1.06x PEM I do the following.

1. Add 4.47 mL of water to a centrifuge tube.
2. Add 530 μL of 10x PEM to the same centrifuge tube.
3. Vortex.

Any left over 1.06x PEM not used in the suspension of GTP or preparation of TSB is discarded.

$MgCl_2$ The $MgCl_2$ used for TSB is the same used in the PEM preparation. I suppose that for absolute completeness I should increase the concentration of PEM used in TSB from 1.06 to 1.061. This is due to the fact that $MgCl_2$ is already in a solution of water and by adding it, I am diluting TSB. However, the dilution by adding $MgCl_2$ is 0.1% and I have not observed any adverse effects from not adding the extra PEM to TSB due to the $MgCl_2$ used. As before, $MgCl_2$ is stored at room temperature in its original container.

PEM-G PEM-G is 100 mM GTP in 1.06x PEM. The reason as to why I put 100 mM GTP in a 1.06x concentrated solution of PEM and not the 1x PEM is because the GTP will be used exclusively in the TSB. As mentioned above, TSB gets diluted by 6% (v/v) when adding glycerol. I have used both a diluted PEM solution and one that takes into consideration the dilution of 6% when adding the glycerol. Both work fine in polymerizing microtubules. To prepare the GTP in 1.06x PEM I follow the below recipe.

1. 1.06x PEM
2. 191.14 μL 1.06x PEM is added directly to the GTP bottle.
3. Vortex the solution and transfer to a screw top vial
4. Flash freeze and store in the -80°C freezer.

The PEM-G solution will last for 6 months in the -80°C freezer.

Glycerol Glycerol can be stored at room temperature and in its original container.

TSB recipe

I prepare a 2 mL solutions of TSB since the smallest amount of glycerol I can measure efficiently is 100 μL. If stored properly in the -80°C freezer, TSB will last up to 6 months. The recipe is below.

- 1858μL 1.06x PEM.
- 6% (v/v) of glycerol which is 120 μL of the glycerol stock.
- 1 mM GTP which is 20 μL of the PEM-G stock.
- 2 μL $MgCl_2$ from stock.

Once I have the TSB prepared, I am ready to prepare aliquots of tubulin that can be used for polymerization into microtubules.

Un-labeled tubulin suspension

Un-labeled tubulin comes packed in vials containing 1 mg of tubulin. I suspend this tubulin to a final concentration of 5 mg/mL in TSB in convenient aliquots.

1. A vial of unlabeled tubulin is removed from the -80°C freezer and put it in the e•IceBucket to defrost. If necessary, I will spin the vial to get all the tubulin to settle at the bottom. Tubulin is very labile and may be destroyed during this step so care should be taken when spinning.

2. Add 200 μL of TSB. I mix the solution by gently drawing the tubulin + TSB mixture back into the pipettor and blow it out again into the vial.

3. The tubulin in TSB is then aliquoted into 5 μL aliquots in 200 μL microcentrifuge tubes, flash frozen in liquid nitrogen (LN_2) and stored in the -80°C freezer.

Labeled tubulin suspension

Rhodamine labeled tubulin and fluorescein labeled tubulin come packed in vials containing 20 μg of labeled tubulin. I suspend these tubulin to a final concentration of 5 mg/mL.

1. A vial of tubulin is removed from the -80°C freezer and put it in the e•IceBucket to defrost. If necessary, I will spin the vial to get all the tubulin to settle at the bottom. Tubulin is very labile and may be destroyed during this step so care should be taken when spinning.

2. Add 4 μL of TSB. I mix the solution by gently drawing the tubulin + TSB mixture back into the pipettor and blow it out again into the vial.

3. I then aliquot into 2 μL aliquots in 200 μL microcentrifuge tubes, then flash frozen in LN_2 and then stored in the -80°C freezer.

29% Labeled tubulin suspension

Using 100% rhodamine labeled tubulin in an experiment is not ideal due to its cost and the possible interference of rhodamine molecules while kinesin is walking along microtubules. 29% rhodamine labeled tubulin to 71% unlabeled tubulin gives a great signal to noise ratio and the microtubules can be tracked easily using the 100 W Hg lamp at 6% illumination. Both the unlabeled and labeled tubulin are now in convenient aliquots at the same concentration of protein in solution. This makes for easy preparation of 29% labeled to 71% unlabeled tubulin. 29% rhodamine labeled suspensions are prepared as follows.

1. Thaw one aliquot of the rhodamine labeled tubulin and one aliquot of the unlabeled tubulin in the e•IceBucket or at 4°C.

2. Add the unlabeled tubulin to the labeled tubulin.

3. Mix gently with the pipettor by drawing the fluid in and out of it several times.

4. 1 μL aliquots of this solution are then flash frozen and then stored in the -80°C freezer.

The 29% labeled tubulin suspension are what I use in experiments. See below for a description of how I polymerize the tubulin into microtubules.

2.2.5 Taxol

Taxol is essential for stabilizing microtubules. However, it is not the only type of "fixing" agent. For an example of experiments using gluteraldehyde as the fixing agent, see Brown et al. [Brown 2002]. I have not performed any experiments using gluteraldehyde.

Taxol overview

Taxol is an anti-cancer drug [Yvon 1999] derived from the Yew tree [Arnal 1995] that stabilizes microtubules. Since cancer cells are fast growing cells, Taxol helps to slow down the spread of the tumor by inhibiting microtubule dynamics and thus cellular replication. The stabilizing effect of Taxol is a good thing for gliding motility experiments as it prevents depolymerization found in Figure 2.4. This type of depolymerization would wreak havoc on tracking in the gliding motility assay and hence the reason for using Taxol. Taxol binds to the inner portion of the microtubule on the beta tubulin subunit ([Nogales 1995], [Arnal 1995]) of a tubulin heterodimer and does so in a stoichiometric ratio to tubulin.

Taxol is hydrophobic so it will not go into an aqueous environment unless the solution has Dimethyl sulfoxide (DMSO) in it. DMSO is an organic solvent that can help Taxol be soluble in PEM. An interesting side note about DMSO is that if it gets on your skin, you will taste garlic almost immediately. Sigma packs DMSO in ampules and the liquid needs to be transferred into a different screw top container for easy access. Since DMSO reacts with just about everything, it must be stored in either HDPE, LDPE, or PP. The screw top cryo vials from Nalgene can be used for DMSO storage. I store the aliquots of DMSO

(Depolymerization movie.)

Figure 2.4: Click here to view the movie via YouTube. Click the movie to play, press P to pause. Movie showing the depolymerization of microtubules.

in a secondary HDPE container filled with desiccant, in a nitrogen environment, and in the desiccator at room temperature.

Preparation of the Taxol solution requires the following components.

- Taxol
- DMSO

Taxol recipe

Taxol is purchased from Cytoskeleton and has approximately 170 μg of Taxol in the vials. Adding 20 μL of DMSO will make a 10 mM Taxol in DMSO solution.

1. One vial of Taxol from Cytoskeleton.
2. 20 μL of DMSO.
3. Mix and store at 3 — 4°C.

(Movie of Taxol crystals in solution.)

Figure 2.5: Click here to view the movie via YouTube. Click the movie to play, press P to pause. Movie showing Taxol crystals found in one of my assays. Note that the crystal does not photobleach.

DMSO freezes around 18°C and is very hygroscopic. Storing the Taxol solution in DMSO at or below 4°C (a refrigerator) is a nice way to check if the solution is still good or not. If the solution freezes, then it is still viable. If it does not freeze, then the DMSO has absorbed enough water from the atmosphere to render it unreliable for experiments.

If there is too much Taxol in solution or, if the Taxol stock has gone bad, Taxol crystals will form. These crystals occur because free tubulin and rhodamine dye molecules have a high affinity for Taxol ([Foss 2008], [Castro 2009], [Castro 2010]). If this occurs in my assays (see Figure 2.5), I will make a new stock solution of Taxol and prepare a new assay. I have noticed that if I use Taxol solutions in DMSO that do not freeze at 3°C, the prevalence of these crystals increases. So, to prevent Taxol crystals from forming in my assays, I always prepare fresh Taxol suspensions and dispose of the stock solution when it no longer freezes at 3°C.

There are some things to take note of about the Taxol crystal in Figure 2.5. The first thing is that the fibers are rigid. If I observe what I think is a microtubule but it does not bend at all, then it is more than likely a Taxol crystal. Also, the crystal does not photobleach very much.

I would also like to point out that when initially running these assays, I used the suggested 2 mM Taxol suspension from Cytoskeleton's website. I noticed that the microtubules fixed with the 2 mM Taxol solution had a greater tendency to depolymerize. I have not run any experiments investigating this but I believe that the reason the microtubules depolymerized more was due to the amount of DMSO in solution. Using the 2 mM stock solution required the addition of more DMSO to solution than the 10 mM stock solution. I have not found any studies investigating this claim so it could have just been my initial inexperience with running these assays that caused the depolymerization to occur when using the 2 mM Taxol solutions.

2.2.6 Kinesin

Kinesin recipe for motility

The kinesin I use was generously supplied by Dr. Haiqing Liu. The kinesin is his-tagged, truncated kinesin-1 dmk401 ([Asbury 2003], [Berliner 1995]); from *Drosophila* and was expressed in *E. coli*.

Kinesin was diluted to 27.5 μg/mL for each assay. In order to prepare the kinesin for an assay from the supplied stock, I have to prepare the following solutions.

PEM-A As mentioned above, ATP is the fuel source for kinesin. It is what kinesin uses in its motor domains to produce a step on a microtubule. In order for the gliding motility assay to work, ATP must be in solution. I make a solution of 100 mM ATP in PEM with the following ingredients.

- PEM
- ATP

ATP comes in different varieties and I always get the Mg-ATP variety because the motility assay requires magnesium in solution. This is why I do not purchase the Na-ATP flavor. Since it is a salt, it goes into a solution of PEM very easily. The stock container of ATP

should be stored in a secondary container that is filled with desiccant and under a nitrogen environment in the -20°C freezer.

The book, *Molecular Cloning* [Sambrook 2001], says to suspend ATP in a Tris buffer at pH 8.0 since ATP auto-hydrolyzes less in alkaline buffers as opposed to acidic ones. Alberty [Alberty 1968] shows a graph that the auto-hydrolysis of ATP doesn't change very much for ATP stored in buffers at pH 7 as opposed to pH 8. Since PEM is pH-ed to 6.89, I figured it would be just fine to store the ATP in it. I have not had any problems using ATP stored in PEM and I believe that not having to introduce another chemical, namely the Tris, into the assay is beneficial. I will note that after storage, the PEM-A solution will start to look cloudy and I do not know the cause of this, however, I have not seen any adverse effects occur to the gliding motility assay from using a solution of ATP in PEM. I will mix the solution as best as I can and spin the precipitate to the bottom of the tube using the mini centrifuge. I'll decant the fluid such that I do not get any of the precipitate in my assays.

To prepare the 1 mL 100 mM PEM-A solution I will do the following.

1. Weigh out 0.0507 g of ATP
2. Dilute the 10x PEM to 1x PEM.
3. Add the ATP to the PEM and mix.
4. Aliquot to 10 μL vials and flash freeze.

PEM-A will last for 6 months if stored in the -80°C freezer.

0.5 mg/mL casein in PEM This is nothing more than a dilution of the 1.0 mg/mL casein solution and is needed for the motility assay.

2.2.7 Flow cell

The above link shows an old method of how I prepared my flow cells. Rather than get rid of it entirely, I have opted to include the link so that it is easier to show the evolution of the flow cell preparation. I put some notes by the video on the subpage pointing out where I did things incorrectly.

Figure 2.6 is a movie (with sound) that outlines my updated version of flow cell creation.

(Movie of flow cell construction.)

Figure 2.6: Click here to view the movie via YouTube. Click the movie to play, press *P* to pause. Movie showing how I make flow cells for the gliding motility assay

2.2.8 Microscope

Images were taken on an Olympus IX71 inverted microscope using an Olympus 60x 1.42 NA PlanApo objective and an Andor Luca S camera via custom LabVIEW image acquisition software [KochLab]. Rhodamine fluorophores attached to tubulin were illuminated with a 100 W mercury lamp using a TRITC filter cube with filter set 49005 from Chroma. The mercury lamp was directly attached to the microscope and attenuated by 94% using neutral density filters. The strong attenuation was to reduce photobleaching and potential local heating of the sample.

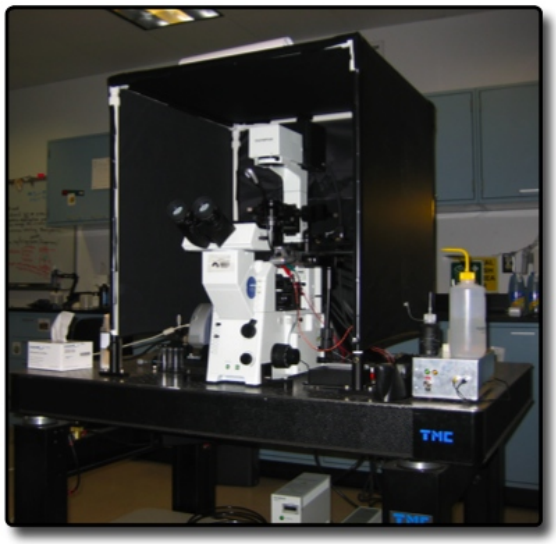

Figure 2.7: Picture of the microscope used for all gliding motility assays.

Temperature stabilization of the objective is crucial for obtaining stable gliding motility

assay speeds. I will describe in detail how I assembled a PID temperature controller in order to stabilize the objective temperature in Chapter 2.

2.2.9 Image acquisition software

An Andor Luca-S camera with custom LabVIEW acquisition software written by Larry Herskowitz was used to acquire images [KochLab]. The acquisition software captures .png files from the camera that are time stamped and stored for later analysis. The camera is attached to a 3 axis stage as well as a rotation stage and not directly attached to the microscope. I found that attempting to center the camera's field of view to the microscope's field of view was nearly impossible if the camera was not detached from the microscope.

2.3 Experiments

In the following section, I will outline how to perform a basic gliding motility assay using the solutions prepared in the above sections. In the following chapters I will change how this basic assay is done and outline what changes were made.

2.3.1 Assay checklist

Before starting an experiment, I always ensure that I have the following solutions and stocks prepared ahead of time.

1. 10x PEM
2. H_2O — just a convenient vial of water is necessary to dilute the 10x PEM to make 1x PEM.
3. 1x PEM
4. Antifade
5. PEM-Glu

6. PEM-A
7. 10 mM Taxol in DMSO
8. 1.0 mg/mL W-PEM
9. 0.5 mg/mL W-PEM
10. 0.5 mg/mL W-PEM + 1 mM ATP (I call this PEM-WA) — This is just 99 μL of 0.5 mg/mL W-PEM and 1μL of PEM-A.
11. 29% labeled, 71% unlabeled tubulin stored in TSB and ready for polymerization.

2.3.2 Microtubule polymerization

The 29% labeled tubulin aliquots are ready for polymerization right out of the freezer. In order to polymerize microtubules, I do the following.

1. Ensure that the thermal cycler is on and set for 37°C. I usually have the lid heated as well since there will be 1 μL of volume in the tube and the heated lid prevents evaporation.
2. The tubulin stays in the thermal cycler for 30 minutes. When 25 minutes have passed, I prepare a solution containing the following which I call PEM-T.
    - 198.8μL of PEM
    - 0.2 μL of the 10 mM Taxol in DMSO.
3. At 30 minutes, I add the PEM-T to the microtubules in the thermal cycler.

The microtubules are then taken out of the thermal cycler and protected from ambient light.

2.3.3 Making a gliding motility assay

Below are the steps I take to make the gliding motility assay.

1. The first step I take is to turn on the mercury lamp and setup the microscope for Köhler illumination. I will then make sure that the camera software is ready to take data. The mercury lamp should be on for at least 30 minutes before taking measurements to ensure that it is warmed up and ready for experiments. Once the microscope is setup, I'll make sure that the temperature controller is on and the software associated with it is working properly.

2. Polymerize microtubules.

3. After microtubules have been polymerized, I add 10 μL of W-PEM to a flow cell and allow it to sit for 10 minutes.

4. Before the 10 minutes are up, I will dilute the kinesin in a ratio of 1:10 Kinesin:PEM-WA and store it in the e•IceBucket until the next step. This diluted solution of kinesin is at a concentration of 27.5 μg/mL.

5. During the 10 minutes of casein incubation, I will prepare a motility solution. Motility solutions consist of

    - 90.5 μL PEM
    - 1 μL PEM-Glu
    - 1 μL PEM-A
    - 2.5 μL Antifade
    - 0.1 μL Taxol
    - 5 μL of microtubules

6. Once the 10 minutes are up, I will add the diluted kinesin in PEM-WA to the flow cell by fluid exchange. This is then allowed to sit for another 5 minutes.

7. After the 5 minutes are up, I add the motility solution to the flow cell by fluid exchange.

8. I seal the flow cell with nail polish and put it on the microscope to observe the system.

The [**?**] suggests including casein in the motility solution. I do not add casein in the motility solution because I have noticed that microtubules undergo non ideal tracking motion, i.e. microtubules will not move in straight lines. There has been some debate about the use of nail polish as the flow cell sealant due to the organic solvents used in nail polish. I discuss the usage of nail polish in detail in Chapter 3.

2.4 Data analysis

Data analysis is performed in the same way for each experiment. I discuss the data analysis in detail in Chapter Chapter 2.

2.5 Conclusion

The gliding motility assay is fickle to put it politely. It is full of possible issues that can cause the completed flow cell to not exhibit motility. This is the worst case scenario as it takes anywhere from 1 to 2 hours to produce the first flow cell of the day. The above recipe works well for me and it has allowed me to create checks with chemical aliquots that help prevent failure of an experiment.

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2.6 Appendix

2.6.1 Quick reference materials list

Below is a quick reference list of the items used in this assay.

Chemicals

- PIPES
- EGTA
- $MgCl_2$
- NaOH
- Catylase
- Glucose Oxidase
- β-mercaptoethanol
- D-glucose
- Un-labelled tubulin
- Rhodamine labelled tubulin
- GTP
- Glycerol
- Taxol
- DMSO
- α-casein

Equipment

- Microscope
- -80°C freezer
- Hot plate/Stirrer
- Fridge
- -20°C freezer
- pH meter
- Electronic Ice Bucket
- Analytical balance
- Thermal cycler
- Autoclave
- Minicentrifuge
- Barnstead Easypure RoDI

Supplies & Tools

- Pipette tips
- Pipettors
- Microcentrifuge tubes
- Microscope slides
- Microscope slips
- Double stick tape
- Tissues

- Weigh boats
- Weigh paper
- Razor blades
- Box cutter
- PP jars
- Screw top vials
- Centrifuge tubes
- Parafilm
- Beakers
- Gloves
- Chemistry clamps
- Clamp holder
- Support stand
- Condenser
- Flask
- Stir bars
- Syringes
- Syringe filters
- Small and Large freezer storage boxes.
- Desiccator
- Slide box