Comparing the strength of non-acetylated tubulin against acetylated tubulin.

James Greene

Independent Research Project Report

Bio254 - Developmental Biology

May 3, 2012

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Introduction

Tubulin is one of several classes of proteins. The most common types of tubulin found in all organisms are α-tubulin and β-tubulin. These two proteins polymerize to make microtubules. Microtubules are found in all cells. (Williams et al, 1999)

Microtubules are rigid hollow rods that are found in all cells and contribute to the cytoskeleton. The can undergo continuous assembly and disassembly in the cell. They function to both the shape of the cell as well as the, the transport of organelles, the separation of cells during mitosis and in the locomotion of the cell in cilia. (Cooper, 2000)

There are two types of cilia, non-motile cilia and motile cilia. Non-motile cilia are used for chemical sensation, signal transduction, and control of cell growth, and are usually only found one per cell. Motile cilia are used for locomotion or for moving liquid over its surface. (Gardener et al, 2006) Almost every cell has motile cilia, including sperm cells, which use their cilia to effectively "swim" up the fallopian tube to the egg to fertilize it.

Sperm cilia contain both tubulin and acetylated tubulin. Acetylated tubulin is tubulin that has been stabilized by a process called acetylation. Acetylation is when an acetyl group is introduced into a compound, usually in place of a hydrogen molecule. When tubulin is acetylated, it stabilizes it so that it won't break down. These acetylated tubulins are found mostly in cilia of organisms, as it is more stable under depolymerizing conditions than the tubulin that is found in cells. (Piperno et al, 1987)

Because tubulin is stabilized by acetylation, that should make it stronger because it won't be depolymerized in cells. Therefore, when sperm cilia are disturbed, the acetylated tubulin should be in larger fragments, than tubulin that is unacetylated.

Methodology

Methanol Fixation

First the embryos were fixed using a MeOH buffer. The buffer was chilled at -80C for over 30 minutes. Then the live embryos were spun down in a 15 ml tube using a hand centrifuge for approximately 10 seconds. The waste was then aspirated off. Then the pellet was resuspended. 15ml of cold MeOH was then poured into the tube with .5ml of live resuspended embryos. The Embryos were then put in the freezer for storage. (R.L.Morris, 2012)

Rehydration

A sample of sperm and egg cells were retrieved from the MeOH fixation and transfered to a 1.5ml Eppendorf centrifuge tube (epitube). The embryos were then allowed to settle into a pellet in the bottom of the tube then the MeOH was aspirated off into a waste beaker, leaving behind the pellet of cells. The epitube was then filled with PBS-T. The cells were left in the PBS-T for 15 minutes to rehydrate. (R.L.Morris, 2012)

Mounting

3 coverslips were stirred around in 100% ethanol and were set on a kimwipe to dry. A humidity chamber was constructed using a moist kimwipe on the lid, parafilm lining the bottom, and the epitube tops to function as pedestals for the coverslips. A bead of about 500 microliters of polylysine solution was spread and let sit on the coverslips for about 60 minutes. The coverslip was tipped onto a kimwipe to remove the polylysine, then rinsed with distilled water and allowed to air dry on the pedestal. The pellet was divided between the three coverslips with a transfer pipette. The cells were allowed to settle and block buffer was added to the cells on the coverslip. (R.L.Morris, 2012)

Blocking

Enough buffer was added so that there was a large meniscus on top of the coverslip. After 5 minutes, the block buffer was wicked off with a kimwipe and new buffer was added. the lid of the humidity chamber was applied and the cells were allowed to block for at least an hour. (R.L.Morris, 2012)

Labeling with Primary Antibody

On the negative control coverslip, most of the block buffer was withdrawn and fresh block buffer was added. (Negative control was used to see if secondary antibody would bind to the primary or to the cells themselves) On the experimentals the block buffer was removed and 500 microliters of a 1:1000 solution of anti-acetlyated tubulin Ab was added. Then the lid was put on the humidity chamber and allowed to sit for at least an hour. (R.L.Morris, 2012)

Washing off the Primary Antibody.

After the incubation was completed, the antibody was wicked off the coverslips and placed in PBS-T rinsing buffer. The coverslip was allowed to sit in the buffer for about 4 minutes before the buffer was exchanged for new buffer. This wash was completed 3 times. (R.L.Morris, 2012)

Labeling with the Secondary Antibody

After the embryo was washed a third time, the embryo was placed back in the humidity chamber and had 250 microliters of 1:2000 Alexafluor 546 Goat anti-mouse secondary Ab to all of the coverslips. Since this antibody is light sensitive, a foil covering was applied to the top to keep light from entering the chamber. They were incubated for 24 hours in the refrigerator. (R.L.Morris, 2012)

Washing off the secondary Antibody

After the incubation was completed, the antibody was wicked off of the coverslip and the coverslip was put in PBS-T buffer for 4 minutes. After the 4 minutes, the PBS-T was removed and fresh PBS-T was added. This was repeated 3 times. (R.L.Morris, 2012)

Labeling with the Third Antibody

After the third wash, the wash solution was discarded and the coverslips were moved to the humidity chamber. 250 microliters of 1:50 FITC-conjugated DM1A Primary Antibody was added to the experimental coverslips. Foil was placed over the chamber, as the antibody is light sensitive. The coverslips were allowed to incubate for an hour at room temperature. (R.L.Morris, 2012)

Washing off with Hoescht

After theincubation, the coverslips were washed off in the 6-well plate with block buffer, plus 1:10,000 solution of Hoescht. The coverslips were allowed to wash for 15 minutes. After 15 minutes, the block buffer was removed and fresh PBS-T was added to the plates. The PBS-T wash was repeated 3 times. (R.L.Morris, 2012)

Mounting on a Slide

Coverslip ships were set in a ring on each of three slides. A small droplet of block buffer was added

in the center of the ring, and the coverslip was set, cells side down, on the slide. This was repeated with the other two coverslips. The slides were then labeled and sealed with nail polish. (R.L.Morris, 2012)

Imaging

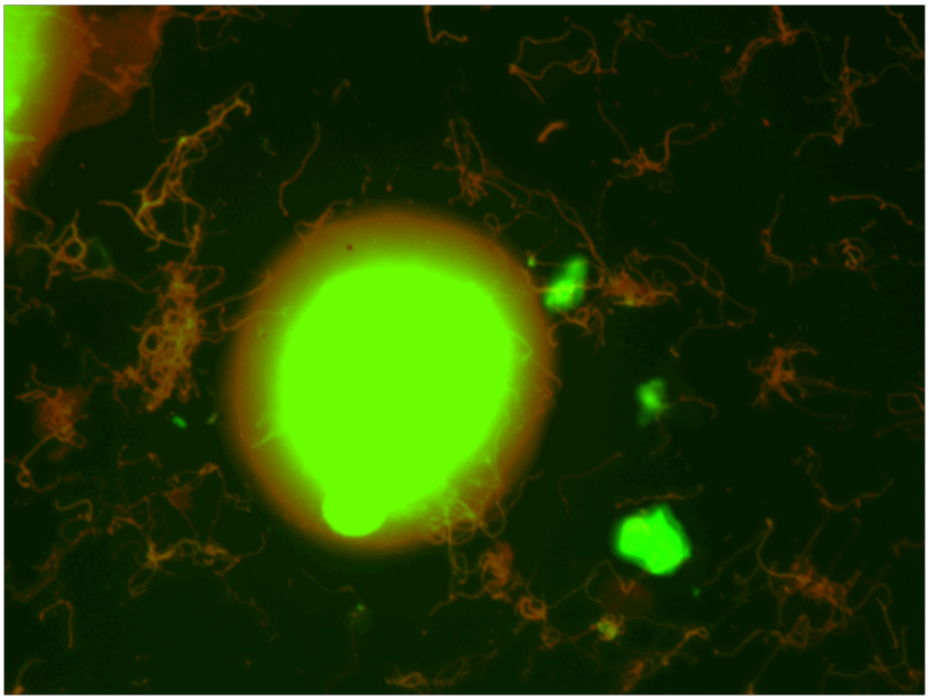
Imaging was done with the program spotify. Prime locations for images were places in which there were only one or two eggs in the frame. DNA, acetylated tubulin, and α-tubulin immunofluorescence images were taken, and then overlain using the program photoshop to combine all three images into one RGB image

Data Collection:

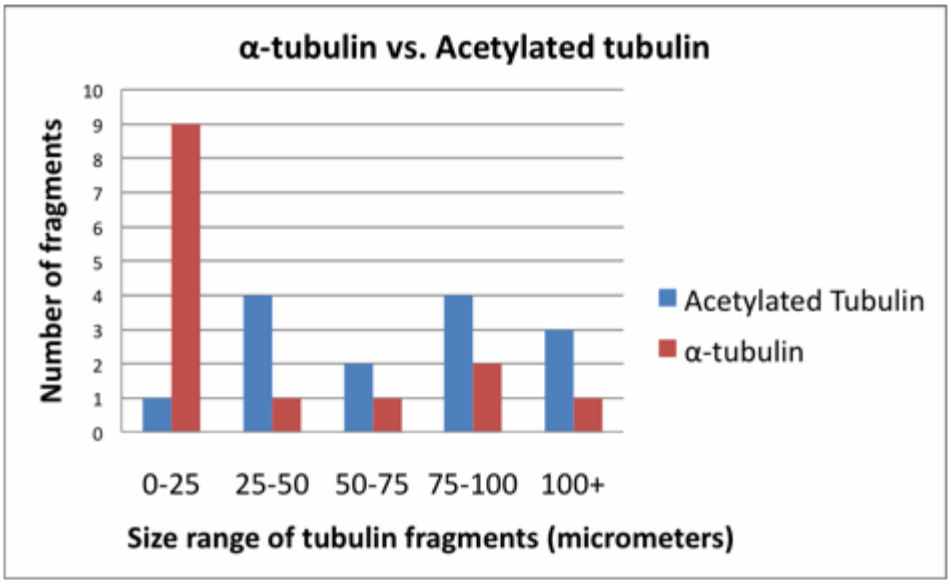
Using ImageJ's line measuring tool, the length of α-tubulin, green, and acetylated tubulin, red, was measured in pixels then converted to micrometers. (.73 micrometers per pixel) 28 data points were taken, 14 of each independent red and green fragment.

Results

In the immunofluorescence imaging, the tubulin was colored green and the acetylated tubulin was colored green. Where the tubulin and the acetylated tubulin are overlapping, there is a yellowish-orange color as seen below.



Where there was only α-tubulin, there is only green, and where there is only acetylated tubulin, those areas showed up as only red. A lot of the sperm cilia were made up of both α-tubulin and acetylated tubulin. However there were a lot of fragments of cilia from the sperm tails that were destroyed in the process of making the slides. By measuring the length of the different fragments, the data showed that on average the acetylated tubulin fragments were longer than the α-tubulin fragments.



The there were a lot more α-tubulin fragments that were 0-25 micrometers in length compared to the

other data sets in which the Acetylated tubulin was predominant.

Discussion

The α-tubulin fragments were indeed on average shorter than the acetylated-tubulin, which is what was hypothesized earlier in the paper. There were far more fragments in the lengths of 0-25 micrometers than there were of any other data range. These results were expected as the acetylated tubulin has been stabilized to prevent them from being disassembled; therefore they should be stronger and less likely to break from stress.

The reason that a red-green overlay was used instead of a three-color overlay was because the pattern of the DNA of the cells was not important to the results. Only the comparison of the α-tubulin and the acetylated tubulin was needed.

Unfortunately, there were only about 14 data points that could be used as there was a lot of complete overlapping between the α-tubulin and the acetylated tubulin. Also the clumping of the sperm cells made it difficult to tell whether a whole flagellum belonged to only one sperm or multiple sperm. The measuring of the data may have been somewhat skewed as ImageJ, the program used to analyze the data, only allowed measuring in straight lines.

Issues with the experiment were that there were not enough data points of just acetylated or just non-acetylated fragments; perhaps due to the fact that the green fluorescence was a lot dimmer than the red fluorescence. It was hard to tell where the α-tubulin fragments were. Future experiments on this topic should be sure to take enough images to gather a larger data set so that results can be more concrete.

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