Total amount of acetylated tubulin in sea urchin cilia of varying lengths

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Introduction:
Cilia are easily observable membrane-bound microtubule projections from a cell. The point from which a cilium projects away from the cell is referred to as the basal body, a structure of triplet microtubules that anchor the cilium (Hammond, Cai, and Verhey, 2008). The building blocks used to construct the extending cilium are transported from the base toward the protruding (+) end in a process called intraflagellar transport (Eggenschwiler and Anderson, 2007). The constructed microtubules are arranged in two patterns, either 9+2 meaning nine outer doublet microtubules surrounding two central microtubules or 9+0 meaning nine outer doublet microtubules organized in the same manner but without the central pair (Satir, 2005). The microtubules in cilia are known to be more stable—resistant to depolymerization, the breaking down of microtubules—than those of the cellular cytoskeleton due to post-translational modifications (Hammond et al., 2008). Post-translational modifications generally occur in polymerized tubulin, tubulin already assembled in a microtubule (Hammond et al., 2008). One post-translational modification that stabilizes microtubules is acetylation the binding of acetyl groups to α-tubulin (Westermann and Weber, 2003). Studies have not found that acetylation of α-tubulin is necessary for cell survival but allows for many specialized functions (Westermann and Weber, 2003).

It was believed that 9+2 cilia had significant function in organisms and 9+0 (primary) cilia were vestigial because they generally lacked the dynein motors necessary for cilium movement (Satir and Christensen, 2007). Recent studies have identified the prevalence of primary cilia throughout the body and the ability of some primary cilia to be motile (Satir and Christensen, 2007). This knowledge instigated investigations of the purpose of primary cilia. Data, found when studying the membrane of cilia, supports that cilia can act as receptors for factors influencing cell growth, cell division, and maintenance of a differentiated state (Satir and Christensen, 2007). There is also evidence that Hedgehog and Wnt signaling pathways during development act through cilia (Satir and Christensen, 2007). Much of this evidence was acquired by identifying abnormal Hedgehog and Wnt signaling in individuals with conditions known to be caused by cilia defects such as polycystic kidney disease, certain cancers, and retinal degeneration (Satir and Christensen, 2007). Therefore better understanding of the structure and receptive functions of cilia could lead to breakthrough treatments of these disorders and pathologies. Defects causing a cilium to be unstable, more likely to depolymerize, would inhibit that cilia’s ability to move or to act as a receptor. A cilium needs enough acetylation to stabilize its structure allowing it to properly function. It is hypothesized that the total amount of acetylated tubulin within a cilium increases with increased length of the cilium. Since sea urchin development is easily-observable and there are cilia present on the cells it can be used as a model organism to study the structure of cilia. In this study, cilia on sea urchin embryos were immunofluorescently labeled to detect the amount of acetylated tubulin in each cilium.

Materials and Methods:

Sea urchin blastulae were immunofluorescently labeled using Robert Morris’ procedure from http://icuc.wheatonma.edu/bio254/2012/rmorris/Immunofluor_Protocol_2012.htm with the following exceptions identified by section.

Antibodies

The FITC-DM1A anti-alpha tubulin Ab solution was prepared to a 1:50 concentration. The Alexfluor 546 Goat anti-mouse secondary Ab solution was prepared to a 1:1000 concentration (Lab Notebook).

Mounting the embryos

A small amount of polylysine was dispensed into three wells of a 6-well plate and the baked coverslips were laid on top of this thin layer of polylysine for 60 minutes. In order to remove the coverslips from the polylysine, they were floated by pipetting PBS-T under the coverslip until it was resting on a meniscus of liquid. The coverslips were then
placed in the other three wells of the 6-well plate, each of which contained 2 mL of block buffer, 3% BSA in PBS-T, which occupied all nonspecific protein binding sites in the blastulae so that the added antibodies would only bind to specific target protein binding sites. Whenever a stop was necessary in the protocol due to class period fluctuations the coverslips were placed in a 6 well plate with 2 mL block buffer and refrigerated (Lab Notebook).

**Label with 1° Monoclonal Antiacetylated Antibody and wash**

This antibody was applied to all three coverslips and the length of time the coverslips were exposed to the antibody was recorded (Lab Notebook).

**Washing excess antibody**

Three wells in the 6 well plate were filled with 2 mL PBS-T. Each coverslip was picked up with forceps and the excess antibody was removed with a kimwipe. The coverslip was gently slid under the PBS-T and allowed to sit for four minutes. This wash was repeated for a total of three washes. A control coverslip was selected and its pedestal and well were labeled. Once the washes were completed the coverslips were returned to their pedestals (Lab Notebook).

**Imaging/Analysis**

The images of each antibody stain were collected on a Nikon Eclipse 80i with a mounted camera using Spot by only exposing the embryos to the light long enough to take a picture in order to not bleach the embryos. The cilia chosen for analysis did not overlap any other cilia to ensure that only the acetylated tubulin in each cilium was quantified. The length of each cilium in pixels and its average amount of acetylated tubulin (measured through the average brightness per pixel of the cilium) were determined using ImageJ. The average amount of tubulin was used as a means of identifying the total amount of acetylated tubulin in a cilium. The average amount of acetylated tubulin in the cilium was plotted against the length of the cilium in Microsoft Excel. A linear line of best fit was added to the plot and the R² coefficient was calculated using Excel to quantify the correlation between the variables.

**Results:**

![Figure 1](http://icuc.wheatonma.edu/bio254/2012/everett_wyll/index.htm)  
Figure 1. This figure depicts a sea urchin ciliated blastula with immunofluorescently labeled acetylated tubulin. The acetylated tubulin in the cilia of the blastula emit red light.
Figure 2. This figure shows the average amount of acetylated tubulin, measured through displayed brightness, in twenty non-overlapped cilia of varying length in Figure 1. A linear line of best fit was added to determine if a correlation between the variables was present. Brightness is measured on a scale of 0 to 255, 0 corresponding the brightness of no light and 255 corresponding the brightness white light. In Figure 2, the brightness scale only reaches 180 because no measured cilia displayed brightness greater than this.

The control embryos are not depicted but displayed no fluorescence indicating that the control embryos contained no compounds that would fluoresce without the added antibodies. Each cilium on the blastula in Figure 1 looks like a red string because of the red illumination of the fluorescent acetylated tubulin that is present in the entire length of each cilium. The brighter the red, the more acetylated tubulin is present in that section of the cilium. In each cilium there is one end of the cilium brighter than the other indicating that there is a much greater concentration of acetylated tubulin at either the base or the (+) end of the microtubules.

The data in Figure 2 was collected from twenty cilia in Figure 1. The data depicted in Figure 2 suggest that the amount of acetylated tubulin in a cilium does not directly vary with increased length of the cilium. For each approximate length of cilium there are both high and low average amounts of acetylated tubulin. This lack of correlation is quantitatively seen through the $R^2$ coefficient of 0.0148, which is far lower than 0.5, indicating that the relationship between these variables is not statistically significant. The lack of correlation is seen through the fact that the data points in Figure 2 are very spread apart and do not follow any general pattern.

Discussion:

The data in Figure 2 indicate that there is no relationship between the length of cilium and the amount of acetylated tubulin within that cilium. This data does not support the hypothesis that the total amount of acetylated tubulin in a cilium increases with the length of the cilium. Though the analysis did quantify the average amount of acetylated tubulin, it did not calculate the total amount of acetylated tubulin directly so the data could have been skewed. Due to the widespread variation for all lengths of cilium it is unlikely that even a direct calculation of the total amount of acetylated tubulin would provide data supporting the hypothesis. Also, if more blastulae were labeled with the antibodies and observed it is likely that there would be a greater number of non-overlapped cilia to analyze providing a larger sample size of cilia. But again, because of the great variation in Figure 1 it is unlikely that increasing the number of cilia analyzed would provide data supporting the hypothesis. This data indicates that other features most likely influence acetylation. Further investigations could identify what features correlate with and perhaps initiate acetylation of $\alpha$-tubulin in the microtubules of cilia and what special functions are gained by the modified cilia through these post-translational modifications.

This data gives insight into the mechanism of acetylation of $\alpha$-tubulin. An unknown enzyme acetylates only polymerized tubulin (Hammond et al., 2008). The data indicate that the activity of this enzyme is not solely dependent on the fact that a cilium is growing. The enzyme might exist along the axoneme, not just in the growing end of the cilium, acetylating tubulin due to other factors besides the presence of newly polymerized tubulin. These data provide support to dispel the notion that the enzyme acetylates tubulin only to stabilize the axoneme as a structure; it serves a
greater purpose than to simply hold the cilium together.

It was interesting that one end of each cilium was clearly a brighter red than the opposite end. It is known that there is more tubulin in the base of a cilium due to the structure of the basal body and this structure is known to have many post-translational modifications to produce its function (Hammond et al., 2008). Research tends to focus on the post-translational modifications in the axoneme—the protruding microtubular structure of a cilium—and not in its basal body (Gaertig and Wloga, 2008). Because this basal body acts as the start point of construction for the cilium it would logically follow that this structure would be very stable—resistant to depolymerization. The amount of acetylated tubulin at the base of each cilium could be concentrated in the basal body and not in the extending cilium, the axoneme, as this study assumed. Further investigation could identify that the brightness observed at one end of each cilium is due to acetylation specifically in the basal body.

References:


