Analysis of Immunofluorescence Microscopy Images of Sea Urchin Embryos

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Introduction:

In this study, the process of cilia formation in sea urchin embryos was observed. The patterns of polymerization of alpha, beta, and acetylated tubulin were studied using techniques in immunocytochemistry. Acetylated tubulin is alpha tubulin that has been post-translationally modified so that an acetyl group is attached to the alpha tubulin monomer of polymerized tubulin (Rosenbaum, 1986). This molecule is often found in the cilia of developing mezozoan embryos (Rosenbaum, 1986). If cilia are present in an organism at a given stage of development, then acetylated tubulin will also be present in any test for acetylated tubulin.

The hypothesis for this experiment is that if acetylated tubulin is present, it will be most common during the blastula and gastrula stages of development. This important because the pattern of acetylated tubulin staining corresponds with cilia formation (Rosenbaum 1986). This is significant because this data can enable researchers to observe when cilia formation occurs. This is not only useful to study the behavior of the embryos, but also to observe the phenotype of the organism, and it is useful to study the patterns of gene expression at different stages of development, and to observe each molecule's role in development.

The organism used to test this hypothesis is the white sea urchin, *lytechinus pictus*. It is a good candidate for the study of early development because it is easy to maintain and it readily produces the eggs and sperm needed to study early development (Gilbert, 2006). In this study, preserved *lytechinus pictus* embryos were stained for immunofluorescence by using antibodies conjugated to fluorescent dyes. These antibodies were bound to alpha and acetylated alpha tubulin to determine the presence of cilia and microtubules, and then imaged using fluorescence microscopy.

Materials and Methods:

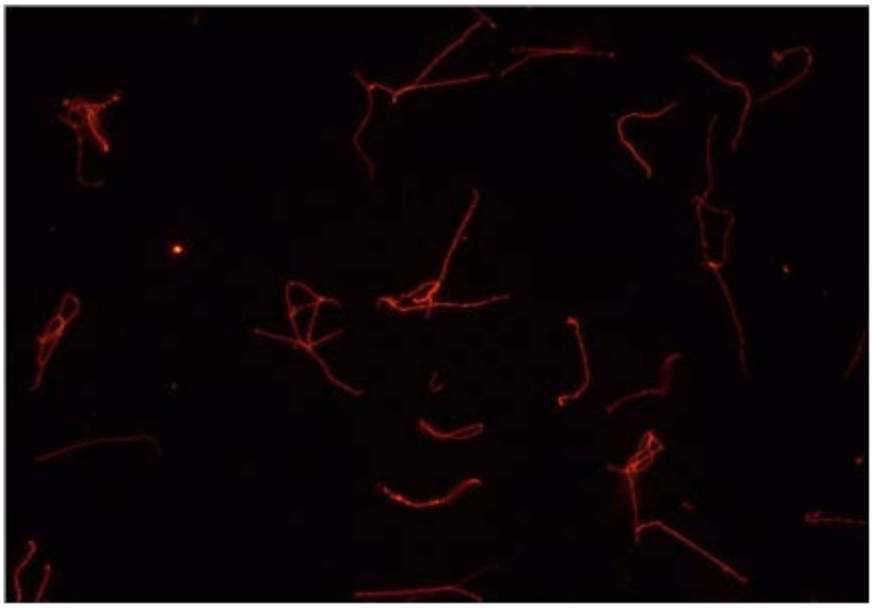
For this study, a protocol for staining for immunofluorescence developed by professor Bob Morris was used. (Morris et. all 2008). However, there were a few minor deviations from this protocol were that the *L. pictus* embryos had been previously been fixed in methanol by Professor Morris. Also, all solutions were prepared previously by

Professor Morris. Also, a protamine sulfate solution was used to adhere the embryos to the coverslips instead of polylysine. Also, three coverslips will be made in this study. The first is the control, and it is treated with secondary antibody and Hoeschst DNA stain only. The two experimental coverslips will be treated with both the primary and secondary antibodies, as well as the Hoechst DNA stain.

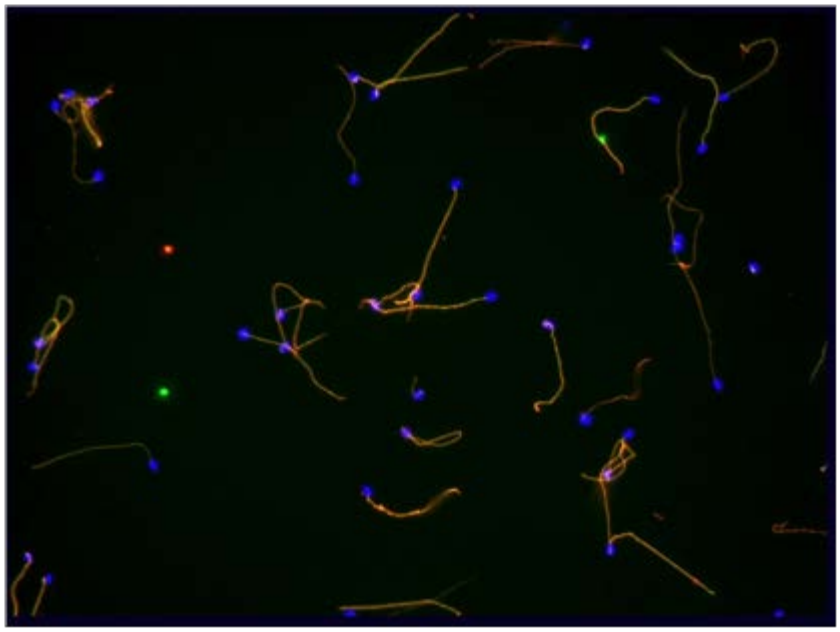
After the staining protocol was followed, the samples were imaged on a Nikon E400 microscope with an epifluorescence attachment with Hoechst, FITC, and Rhodamine filter sets using Spot advanced software on a Spot insight camera made by diagnostic instruments. A 40x plan fluor objective was used for all images.

Results:

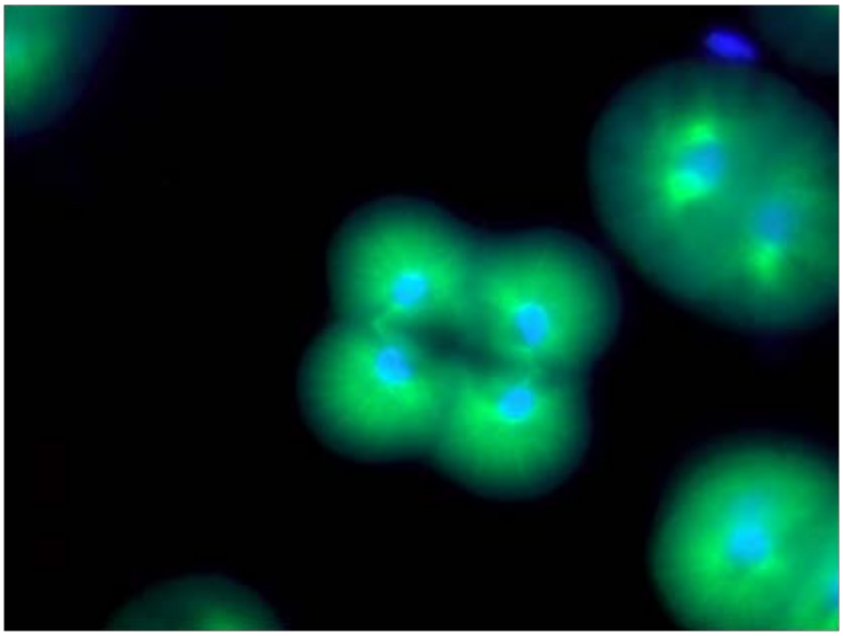
Figure 1: Images captured Using Fluorescence Microscopy on immunofluorescence stained sea urchin embryos at various stages of Development



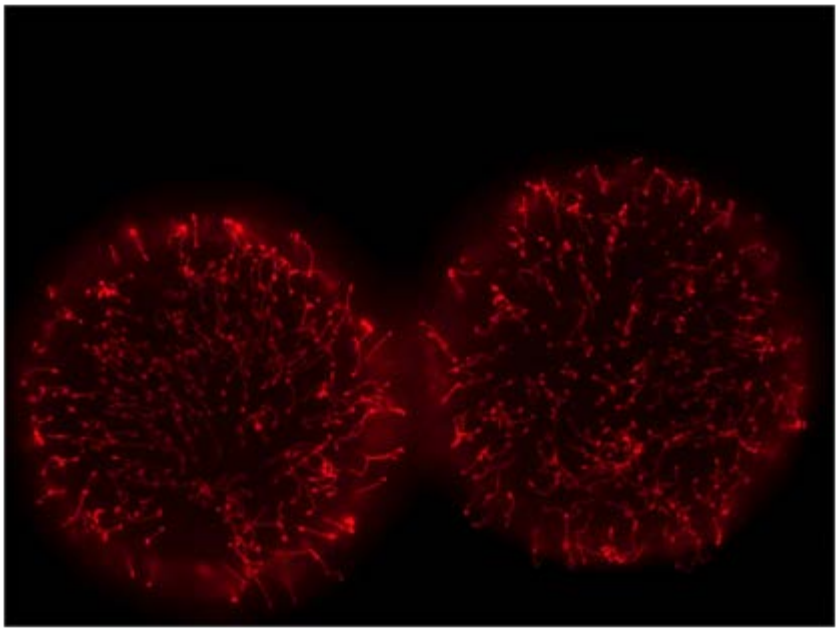
Here, the staining pattern of acetylated tubulin can be seen in the tails of *lytechinus pictus* sperm cells. Only the tails of the cells glow red, because this is the only place that acetylated tubulin is present at this stage of development.



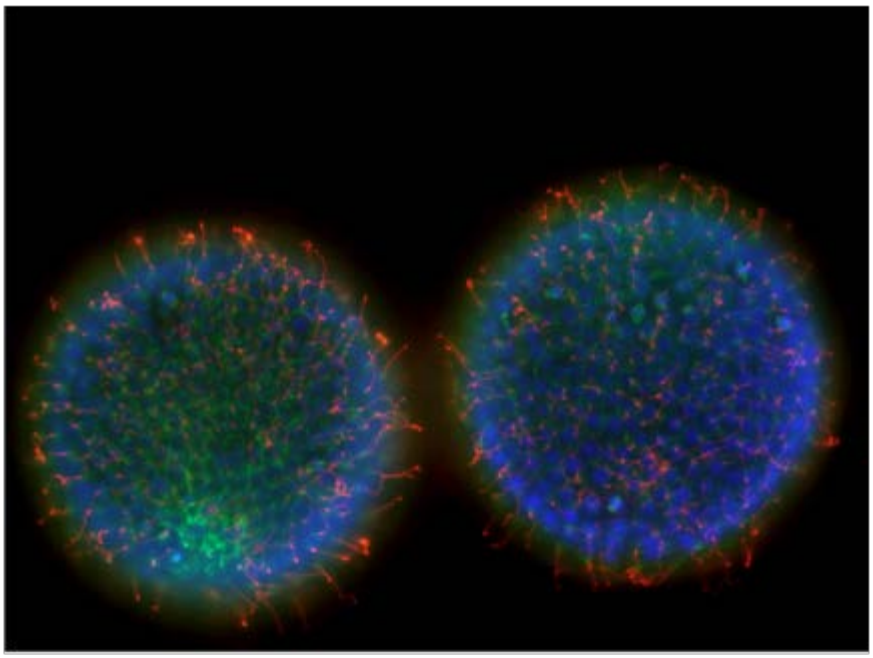
Here, the staining pattern of alpha tubulin, acetylated tubulin, and DNA are all shown in an overlay in the sperm cells of *L. pictus*. The staining patterns of alpha and acetylated tubulin appear yellow when superimposed. The sperm tails contain both alpha and acetylated tubulin, while only DNA appears in this image in the sperm heads.



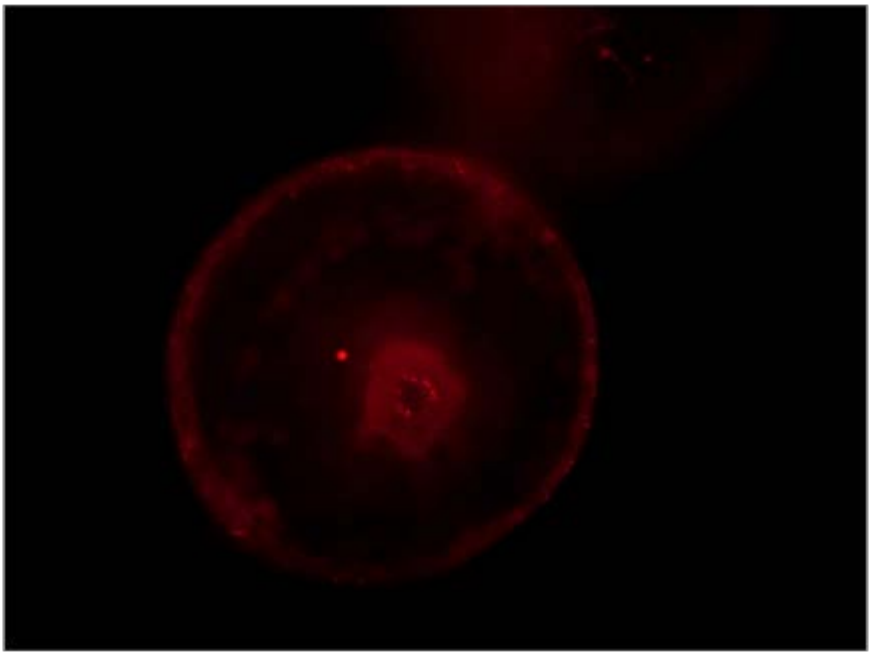
In this image, the *L. pictus* embryo can be seen at the mitosis of the 4 cell stage of development. Here, no red acetylated tubulin appears because none is present during the 4-cell stage of *L. pictus* development. However, the alpha tubulin and DNA staining can readily be seen, and the microtubules can be seen forming around the condensed chromosomes.



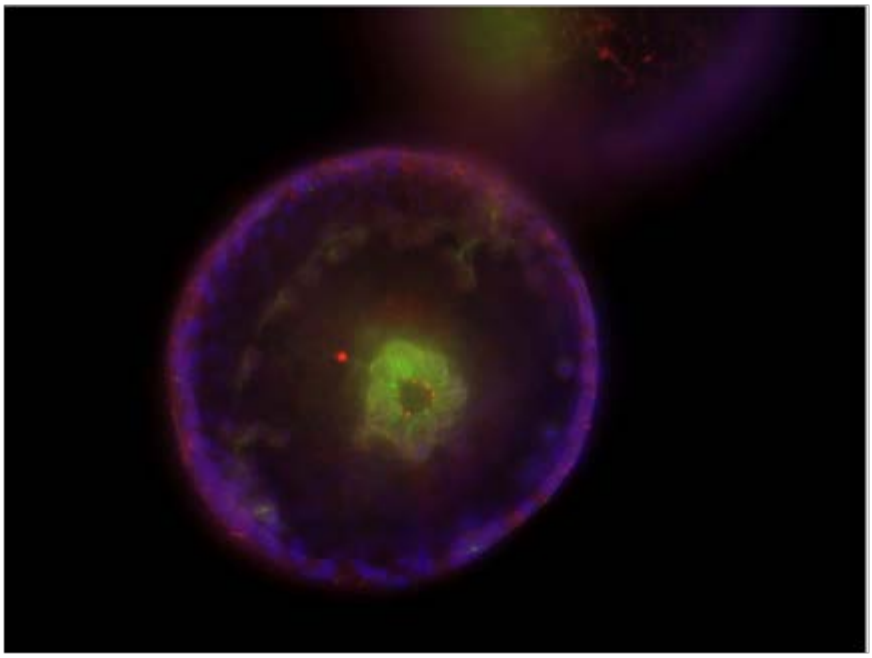
Here, two *L. pictus* embryos can be seen during the blastula stage of development. This is a useful image because it clearly shows the acetylation patterns of the tubulin in cilia. Each small, bright red fiber protruding from the body of the embryo is an individual cilium.



Here, the staining patterns of alpha tubulin, acetylated tubulin, and DNA can be seen in an overlay during the blastula stage of development. This is presented in order to show the pattern of tubulin acetylation and cilia formation in relation to the cells found in the blastula.

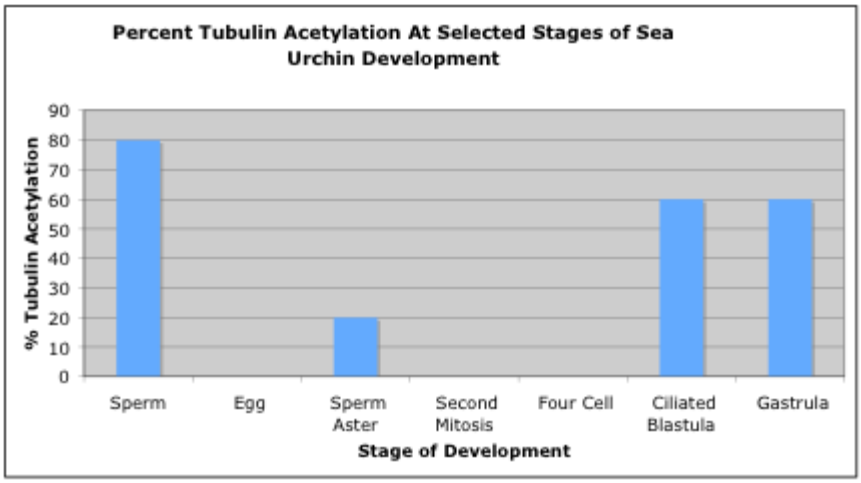


Here, the staining pattern of acetylated tubulin can be observed in the gastrula stage of a *L. pictus* embryo. This is a top-view cross section of the embryo. The small ring in the center is the layer of tissue surrounding the archenteron. The small, bright, red dots on the inside of the archenteron are cilia. These cilia are also present around the ectoderm (The outer layer of cells), but because they are lying flat along the outside of the embryo, they are more difficult to spot.



Here, the same top view of an *L. pictus* embryo can be seen, but the alpha tubulin, acetylated tubulin, and DNA staining patterns are shown in an overlay. This is useful because the cilia protruding into the archenteron and in the ectoderm can be observed in relation to the cells around them. Again, the acetylated tubulin staining pattern in this image is present around the outside the ectoderm and the layer of tissue surrounding the archenteron.

Figure 2: Percent Tubulin Acetylation at Selected Stages of Sea Urchin Development



Here, the relative percentage of tubulin acetylation of each cell of the specimen is shown at each stage of development. During the sperm stage before fertilization, the entire tail of the sperm cell contains acetylated tubulin. During the egg stage, no acetylated tubulin is present. During the sperm aster stage, the tubulin in the tail of the sperm after it enters the egg is still acetylated, but the rest of the fertilized egg does not contain acetylated tubulin. The second mitosis and four-cell stages do not contain any acetylated tubulin staining. The ciliated blastula stage embryo possesses quite a bit of acetylated tubulin, as does the embryo at the gastrula stage of development.

The percentages of tubulin acetylation in this figure is an approximation of the amount of acetylated tubulin in all cells present in the sample. First, it was determined if acetylated tubulin was present in each sample. Once this was determined, an estimate was made as to how much acetylated tubulin was present in comparison with the entire cell. For example, the percentage is highest at the sperm stage because the tail composes a large percentage of the whole sperm cell. The percentage is lowest at the sperm aster stage because only the tail of the sperm contains acetylated tubulin, while the remainder of the cell does not contain any.

Discussion and Conclusions:

The results of this study confirmed the hypothesis for this experiment. Acetylated tubulin was most common during the blastula and gastrula stages of development. This corresponds to the presence of the cilia that formed during blastulation. Additionally, acetylated tubulin was present in the tails of sperm cells. While not predicted in the hypothesis, this result is not surprising at all, due to the fact that sperm tails contain a functional microtubule axoneme, which is required for motile cilia, which is basically what the sperm tail is.

There are several conclusions that can be drawn from this data. First, the data demonstrate that cilia are found most frequently in the sperm, blastulae, and gastrulae phases of developing embryos. Second, the anti-acetylated

tubulin antibodies only bound to cilia in *L. pictus*. Therefore, the evidence suggests that acetylated tubulin is found only in cilia in *L. pictus.* Furthermore, it is possible to conclude that cilia are present in the archenteron of the gastrula, of *L. pictus* embryos. This is extremely interesting because the role of the cilia inside the archenteron is not entirely clear, so further research into their activity could possibly provide some insightful information.

If this experiment had been repeated a thousand times and the same results were obtained from each trial, then it would be possible to conclude that the hypothesis for this experiment applies to all *L. pictus* embryos. It would be safe to conclude that all *L. pictus* sperm, blastulae, and gastrulae contain acetylated tubulin. In addition, it would be safe to conclude that this acetylated tubulin composes the cilia of developing embryos. Therefore, it could be possible to safely conclude that acetylated tubulin is an important component of functional embryonic sea urchin cilia if this data were present.

From a biological standpoint, the test results not only indicated the presence of acetylated tubulin and cilia, but it also showed that the antibody incubation and binding worked. In order for the sample to glow, the primary antibodies for anti-alpha tubulin and anti-acetylated tubulin must successfully bind to their substrates, and the secondary antibody for acetylated tubulin must also successfully bind to its substrate. The substrates for the primary antibodies are alpha tubulin and acetylated tubulin, which are both vital components of functional cilia. The substrate for the secondary antibody is the primary antibody for acetylated tubulin. Because the samples glowed, the presence of the antibodies shows that the staining procedure worked.

The controls from this experiment also showed that the experiment worked. The controls used were the slides that were not incubated with any primary antibodies. Because the secondary antibodies did not bind to anything on the prepared control slides, the control slides exhibited no fluorescence. This meant that the secondary antibodies were binding to the primary antibodies in the experimental slides. This also meant that there was no non-specific antibody binding, which would have altered the results. The fact that the experimental slides exhibited fluorescence and the negative controls did not provides evidence that the experiment was a success.

There were a number of potential sources of error, but because the experiment was relatively successful, it was unlikely that they played a significant role in the results. The first potential source of error is that the incubation chamber could have been set up improperly. If the prepared slides are not stored correctly in a humidity chamber, then it is possible for them to dry out, and the cells become shriveled and they become impossible to image. Other sources of error that could have occurred include errors in making the PBS-t, protamine sulfate, and block buffers by producing them at the incorrect concentrations, applying the antibodies by applying the incorrect antibodies, not allowing the antibodies to incubate for long enough, or exposing the samples to too much light or heat during and after incubation.

Incubation time is especially tricky, as this protocol called for a relatively short incubation time. Immunofluorescence procedures often call for over 24 hours of incubation time for each antibody (Ko, 2009). All of these sources of error could have been detrimental to this study, but there were no noticeable problems with any part of the procedure in this case, so it is likely that none of these sources of error occurred.

Although this study yielded some exciting results, there are several ways to refine this experiment. The first way to refine this experiment would be to use a different imaging system to view the samples. In this study, a Nikon Eclipse E400 light microscope with a fluorescence attachment was used to image the embryos. While this equipment worked well and yielded good results, there are better imaging systems for this purpose. A better piece of equipment for this study would be a confocal microscope. A confocal microscope possesses the ability to collect light in a single plane (Ziess, 2009). This would be extremely useful because the *L. pictus* embryos in this study are spherical, so it is impossible to get an image that is truly focused on a single plane by using a standard light microscope. An image gathered of a spherical embryo with a standard light microscope contains light from multiple planes, which can distort and cloud the image. A confocal microscope would give the viewer a much clearer image of the desired plane. However, confocal microscopes are extremely expensive, so it is understandable why one was not used in this study.

Another way that this experiment could be refined is by using different antibodies for the staining procedure. The antibodies selected for this procedure were designed to bind to alpha tubulin and acetylated tubulin. The antibodies could have been manufactured to bind to other proteins found in cilia, such as motor proteins including kinesins and dyneins, which are present in cilia (Reed 2006). It would be interesting to view the fluorescence staining patterns of motor proteins in addition to alpha and acetylated tubulin, which are structural proteins.

Another way that this experiment could be refined is to have the ability to gather more precise, quantitative data. Due to the nature of this study, it ended up being more of a test for the presence of acetylated tubulin than a quantitative data gathering exercise. It would have been nice to obtain some more precise data than the data that were acquired. However, the results from this study are still very exciting and relevant, and they are a good start if more quantitative data were to be gathered.

There are several future experiments that could be performed in this area that could provide some useful information. First of all, the same experiment could be performed on a different organism, especially one that is quite different from the sea urchin. Some possible candidates could include mammals such as mice. The staining patterns could possibly be different, and it would be very interesting to observe these differences compared to the staining patterns in *L. pictus.*

Another future experiment that could yield some interesting information is the use of different antibodies. These

antibodies could bind to other proteins found in cilia, or even other organelles. By observing the motor proteins in the cilia as well as the acetylated and alpha tubulin, it would be possible to gain an even better perspective of what goes on in cilia during development. It may even be possible to quantify the number and movement of those motor proteins along the microtubules in cilia, or proteins in another organelle could be observed as well.

Sources:

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