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Effect of extracellular ATP on cellular actin fibrils' location and characteristics

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EFFECT OF EXTRACELLULAR ATP ON CELLULAR ACTIN FIBRILS' LOCATION AND CHARACTERISTICS

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Abstract

Epithelial cells lining secretory units and ducts of bovine mammary glands perform an important role in regulating movement of various macromolecules and whole cells during normal lactation and mastitis. During mastitis, host-and bacteria-produced substances can affect the “barrier” function of epithelial monolayers. One potential component is adenosine triphosphate (ATP). ATP likely interacts with P2X7, a purinergic receptor, in mediating some effects associated with mastitis including changes in cell permeability. The bovine mammary gland epithelial cell line, Mac-T cells, were examined for cytoskeletal changes as result of P2X7 interactions. Actin cytoskeletons were stained with phalloidin and effects were examined by fluorescent microscopy. Observable increase in actin fibril size was noted in ATP treated cells. Results indicate the possibility of ATP modulating epithelial cell function in bovine mammary glands affecting the barrier function epithelial cells normally provide, through interaction with the P2X7 receptor.

Acknowledgments

I would like to thank Dr. David McClenahan for acting as my thesis advisor and his help and guidance through the project, Dr. Ali Tabei for assistance in image processing techniques, Madison Shatek for collaboration performing experiments, and Joseph Tibbs for collaboration with image processing. I would also like to thank the College of Humanities Arts and Sciences for awarding me the Student Opportunities for Academic Research (SOAR) award which made this project possible.

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Introduction

Bovine mastitis is an inflammatory condition in the mammary gland caused by bacterial infection. It has great significance for the cattle industry due to its effect on milk and milk products. Bovine mastitis is especially prevalent in developing countries where there is a lack of access to sanitary milking equipment. Bovine mastitis can be used as a disease model of inflammatory conditions of epithelial cells. Elevated levels of extracellular ATP are present in this disease. Lister et. al. (2007) demonstrated that similar elevated levels of extracellular ATP are found in the inflammatory condition asthma, as well as in other airway inflammations.

This project is a study of the effect of ATP (adenosine triphosphate) on cellular actin. Extracellular ATP is considered to be an inflammatory mediator and it has been previously demonstrated by McClenahan et. al. (2009) to have multiple effects on cells including modulating their permeability. To further focus on the permeability aspects, I examined the effect of ATP on the location and characteristics of actin, a known structural component, within the cell. Exposure to extracellular ATP may make actin fibrils more diffuse. Diffuse actin fibrils can be a sign of cellular damage, as a result of inflammation, according to Lister et. al. (2007).

The immune response to extracellular ATP has been studied on the cellular level, but no major studies have been conducted on actin in the cell. My research on the effect on actin is important because of its impact on cellular structure and its potential to modulate cell permeability during inflammation. This research can apply across a large scope of illnesses that cause inflammation. Through this study we can gain a better understanding of the types of changes that occur in a cell under inflammatory conditions.

Previous studies have linked extracellular ATP-P2X7 interactions as an effector of change in a cell's structure and function. With further testing using inhibitors it will likely be possible to determine if the P2X7 receptor is directly responsible for actin changes in a cell, and therefore if the P2X7 receptor is an important component in the inflammatory pathway. If so, the P2X7 receptor could then be targeted for treatments to inflammatory conditions.

Literature Review

The P2X7 receptor has been recognized as an important receptor in the inflammatory response to inflammatory mediators. Lister et. al. (2007) stated that the inflammatory response can occur due to a variety of injurious agents, including bacteria. Also, this inflammatory response is likely in part due to the P2X7 receptor, part of the P2X family of receptors, which are known to be activated by the presence of extracellular ATP.

A variety of studies have been performed on the effect of ATP activation of P2X receptors on the cellular cytoskeleton. Homma et. al. (2007) performed a set of experiments that demonstrated extracellular ATP is capable of increasing calcium uptake, therefore causing rearrangement of the actin cytoskeleton by cofilin rods in neurites. This suggests that the ATP-induced cofilin rods are capable of restructuring the actin cytoskeleton in neurons. In a similar study, Goldman et. al. (2013) examined the effect of purine (the base compound of ATP)-induced Ca^{2+} signaling on actin in the cell. Their research demonstrated that increased Ca^{2+} signaling causes remodeling of the actin cytoskeleton. This phenomenon supports the hypothesis that extracellular ATP exposure can cause remodeling of the actin cytoskeleton, because of the role extracellular actin plays in Ca^{2+} signaling.

Connecting this exposure to extracellular actin to the P2X7 receptor activation and remodeling of actin filaments, Qu and Dubyak (2009), showed the activation of the P2X7 receptors are associated with disruption of actin filaments, allowing for depolymerization. The P2X7 receptor can be activated by ATP, and when activated induces a rapid, sustained calcium influx. This finding when considered along that of Goldman et. al. (2013) encourages the hypothesis that extracellular ATP will cause remodeling of the actin cytoskeleton. Pubill et. al. (2001) demonstrated similar findings that support the hypothesis that extracellular ATP

stimulates actin deorganization through the P2X receptors. This further backs the argument for extracellular ATP causing a locational or characteristic change in intracellular actin. The link between activation of P2X receptors by extracellular ATP and downstream actin remodeling prompted the question of locational or characteristic changes in intracellular actin.

Further research performed by Kolliputi et. al. (2010) demonstrated another effect of extracellular ATP exposure. It was experimentally determined that the activation of the P2X7 receptor led to inflammasome activation and increased permeability of the cell membrane. The inflammasome is a complex that secretes pro-inflammatory cytokines. This finding relates to the state of inflammation occurring in conditions such as asthma and other airway inflammations.

Lister et. al. (2007) demonstrated a similar role by the P2X7 receptor in inflammatory disease such as rheumatoid arthritis, chronic obstructive pulmonary diseases, and atherosclerosis. The experiment suggested that the P2X7 receptor could be regulated by a variety of ligands, including ATP. Many of these effects were reviewed by Gordon (1986) in an article remarking that effects of extracellular ATP include increasing membrane permeability, vasoconstriction and dilation effects, and increased platelet aggregation. It is important to note that his work also examined a variety of sources of extracellular ATP, including cellular degranulation, cell lysis, and release through synapses.

Research Questions

Does exposure to extracellular ATP affect actin fibers in a cell?

Through this study, direct microscopic observation was performed to determine if exposure to extracellular ATP affects actin fibers in the cell.

What are the observed effects of extracellular ATP on actin fibers?

Actin re-distribution within the cell was measured along with filament size using image analysis programs such as ImageJ and DiameterJ.

Does the duration of exposure to extracellular ATP change the effect on actin?

Using the aforementioned technique, the effect of varying duration of ATP exposure on intracellular actin was examined.

Through my study, it was determined if and how actin in a cell is affected by exposure to extracellular ATP when considering its location and characteristics. I then described these effects, and quantified them. Data was used to determine if the effect of exposure to extracellular ATP caused statistically significant change in the actin fibers in a cell. Different durations of exposure were tested, and the relationship between time of exposure and change in actin fiber concentration and diameter were determined. Findings were related to the immunological study of inflammatory mediators by discussing the relation of my project with previous experiments on the permeability changes occurring in these cells.

Materials and Methodology

With the help of Professor David McClenahan and fellow undergraduate researcher Madison Shatek, bovine cells were cultured, and placed in wells on microscope slides. Mac-T cells, a bovine mammary gland epithelial cell line were used in this experiment. The cells were grown in DMEM/F12 with 10% fetal bovine serum. When confluent the cells were detached with trypsin, and seeded into a slide with culture chambers. Slides were incubated at 37°C in 5% CO_2 until cells were confluent, as determined by microscopic examination.

Two wells received vehicle control cells, while other wells received 10 mM of ATP treatment respectively. Incubation at room temperature varied with different amounts of time of exposure to ATP. Four exposure times were used as follows: no treatment, 5 minutes, 15 minutes, and 30 minutes. The media was then removed from wells, wells were detached from the glass slide, and the cells on the slide were fixed using 4% paraformaldehyde, then permeabilized in 0.5% Triton X-100. The cells were washed in PBS, then blocked for an hour with 1% bovine serum albumin in PBS. Cells were incubated with a solution of FITC-phalloidin for 1 hour at room temperature. The slide was washed, then cover slips were placed on the slide to prepare for examination. If needed, slides were stored in a dark box in the refrigerator under humid conditions to prevent drying.

The cells were examined under a fluorescence microscope using the “FITC” filter. ATP treated cells were compared with the vehicle treated cells. Testing was also performed to determine the effect of inhibitors on the cells treated with extracellular ATP. In these experiments prior to exposure to ATP, inhibitors were added to the media. The changes in location and characteristics of the actin fibers were noted.

Changes were quantitated using the ImageJ, DiameterJ, and Matlab programs. Images were processed using a Mexican Hat filter and grayscale, then segmented using DiameterJ, an ImageJ plugin. Several segmented images were produced, and the image that best represents the actin fibers visible in the fluorescent image was chosen. DiameterJ was then used to run a segmentation analysis producing histograms of fiber diameter. Matlab was used to analyze the ratio between black and white pixels in the segmented images produced by DiameterJ, giving an idea of the amount of actin present in each image. Data generated by DiameterJ was examined by statistical analysis using an ANOVA analysis ($P \leq 0.05$) to determine if there were any significant differences between the different groups.

Results

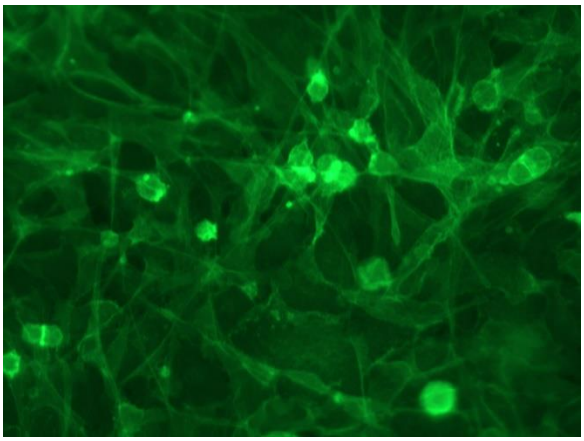
DiameterJ

Treatment vs. Fiber Diameter

Fluorescent images (Figure 1:A) were taken and Mexican Hat Filter and Grayscale were applied (Figure 1:B). DiameterJ was then used to produce a segmented image (Figure 1:C). Segmented images were analyzed to produce histograms of fiber diameter (Figure 1:D). These representative images illustrate the results gained for each trial and treatment. Multiple images were taken at each time point and can be seen in Tables 1 and 2 in the appendix.

Figure 1

A



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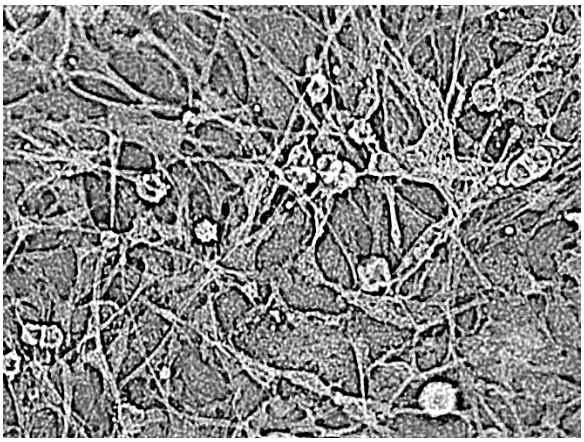

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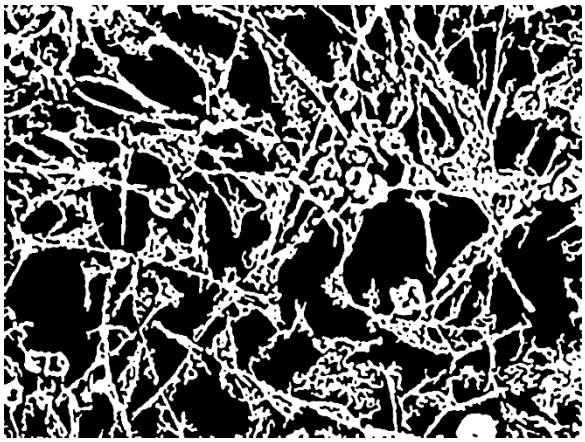

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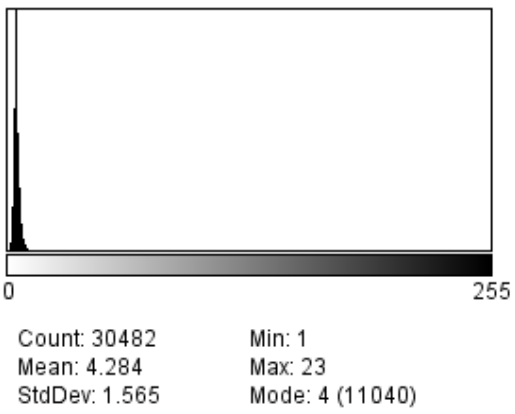
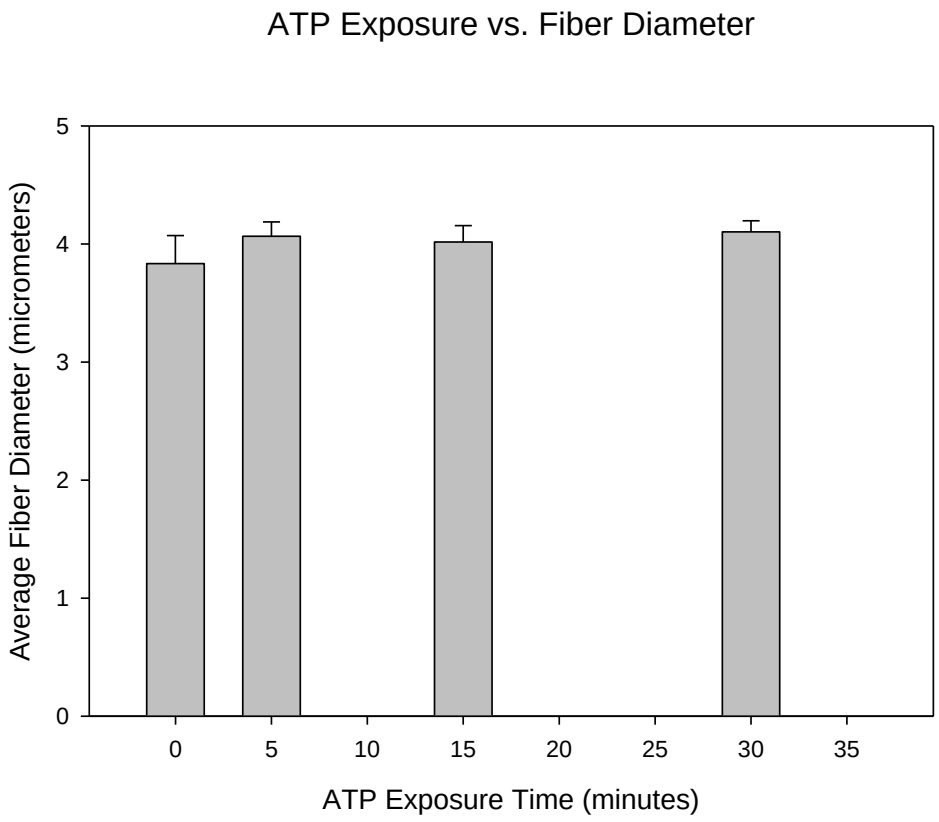


Fiber diameters (Table 2, Appendix) from three sets of experiments were averaged to create the graph below (Figure 2). Bars represent the average ±SEM of the fiber diameters at 0, 5, 15, and 30 minutes exposure to ATP. ANOVA statistical analysis was used to determine that the data was not statistically significant. Error bars in the graph were determined through the ANOVA analysis.

Figure 2

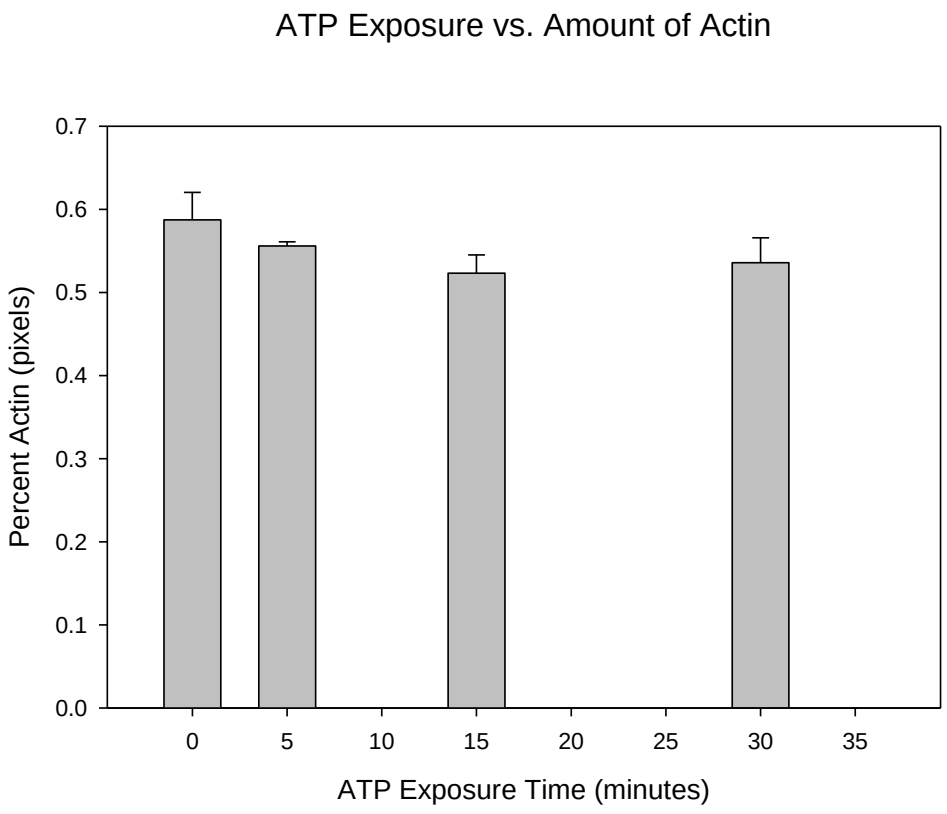


Matlab Analysis

Treatment vs. Percent White Pixels

In order to determine the relationship between exposure to extracellular ATP and the amount of actin present in cells Matlab was used. The percent of black pixels was determined from segmented images (Table 3, Appedix). Percent black pixels were averaged for each time point and then converted to percent white pixels. The graph produced (Figure 3) relates the time of exposure to extracellular ATP to the relative amount of actin at each time point. ANOVA statistical analysis was used to determine that the data was not statistically significant. Error bars in the graph were determined through the ANOVA analysis.

Figure 3



Discussion

Exposure to extracellular ATP appears to create changes in actin fiber characteristics and amount, although not statistically significant. It was observed that during some trials the ATP treatment of 30 minutes caused more diffusion of actin than lesser time treatments. It was also observed that the no treatment trial had the most concentrated actin fibers. During another trial no greater degree of diffusion of actin fibers was observed between the no treatment and 30 minute ATP treatment trials.

It was also observed that actin fibers were spread throughout the cells and not congregated in any specific area of the cell. Actin fibers of the no treatment trials were characterized as defined and spread throughout the cell without specific regional concentrations. These results show that extracellular ATP may have an effect on actin in cells. Extracellular ATP exposure may make actin fibrils more diffuse, a sign of cellular damage as a result of inflammation. These observations lead to further analysis of the actin fiber characteristics and amount by DiameterJ and Matlab.

Through those analyses, it was found that there was no statistically significant change in either fiber diameter or amount of actin present in cells. When examining the data there does appear to be a trend present in both of these characteristics. Previous permeability tests showed a fairly quick effect on permeability followed by a return to normal permeability. A similar trend can be seen in the fiber diameter data, which appears to have an increase in fiber diameter followed by a decrease toward the original fiber diameter average. When examining the actin data regarding the percent of white pixels, there seems to be a decline in the amount of actin followed by a return toward the original percentage.

More trials could provide a clearer picture on whether or not there is a statistically significant trend in this data. Further statistical analysis may also be helpful in understanding the effects extracellular ATP exposure has on actin fibers. An analysis that focuses on the changes in fiber diameter and amount of actin in each experiment rather than averages between experiments may be helpful. Another future interest is in using the confocal microscope to capture these effects in real time using fluorescently labeled cells.

Additional future directions for this research include performing similar analyses on experiments performed with inhibitors to the P2X7 receptor. It is expected that an inhibitor to this receptor will then reverse the effect of extracellular ATP on exposed cells, proving that the change in actin fibers is due to the interaction we hypothesized.

Conclusions

Exposure to extracellular ATP produced an effect on intracellular actin fibers. Changes were observed qualitatively in actin fiber amount and characteristics. These changes are quantifiable and trend toward a decrease in actin fiber amount, with a marked increase in actin fiber diameter. It is hypothesized that this is due to aggregation of actin fibers in the cell, or a change in the actin from the fibrous form to the globular form. Although the observed changes were not statistically significant once quantified, it is possible that another form of statistical analysis may yield a better understanding of the effects on actin in the cell.

When addressing the research questions, we can conclude that exposure to extracellular ATP does affect actin fibers in a cell. It was qualitatively observed that there are differences in visible actin fibers when comparing no treatment cells to cells that did receive exposure to extracellular ATP. These observed effects include changes in fiber diameter, as well as changes in the amount of actin, although both of these trends were determined to not be statistically significant. Lastly, these changes are related to the time of exposure to ATP. It is likely that cells undergo actin remodeling, which is quickly recovered from to return the cell to a normal state.

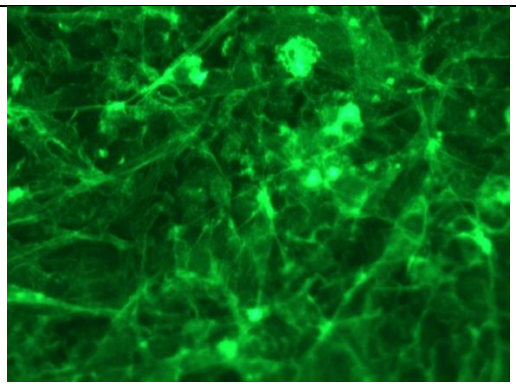
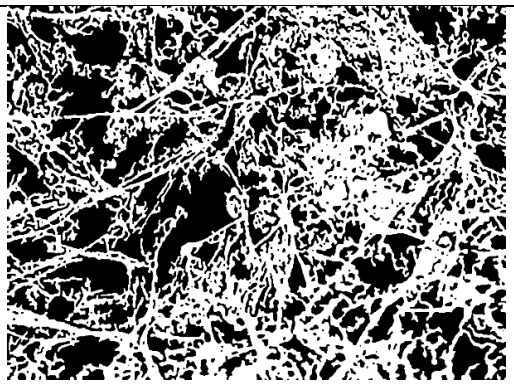
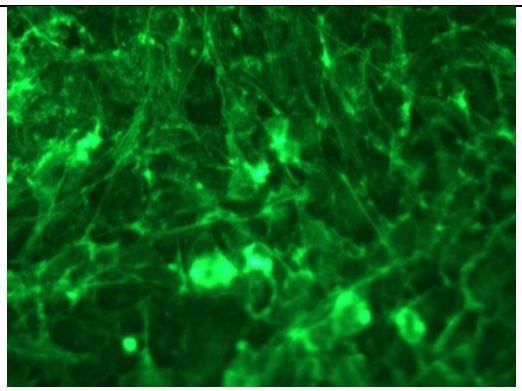
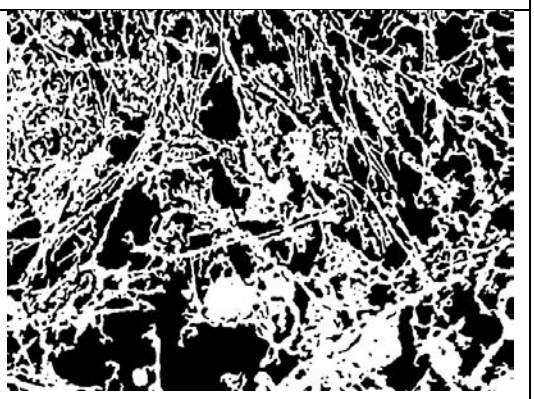
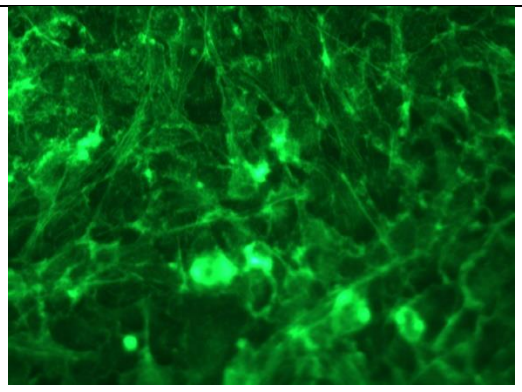
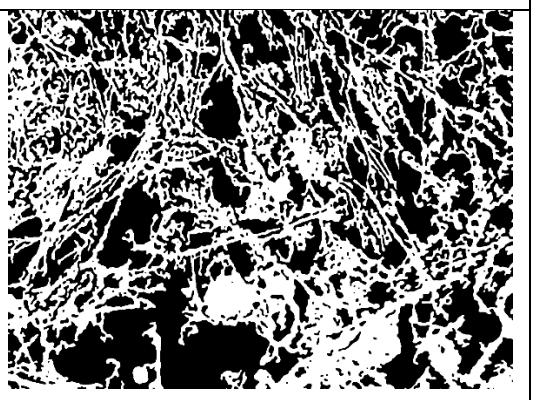
Through this project a better understanding of the effect of inflammatory mediators on a cell was achieved. Actin, a structural component, may be undergoing some important changes in fiber diameter as well as form. Actin remodeling has been demonstrated under inflammatory conditions before, and is a known indicator of damage to a cell. Through examining this actin remodeling under the inflammatory condition of extracellular ATP, a clearer picture of the changes actin undergoes during remodeling is being formed. With further research using inhibitors it is possible to determine whether or not the P2X7 receptor functions in this pathway

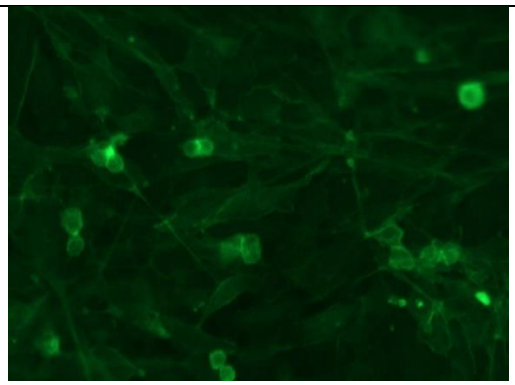
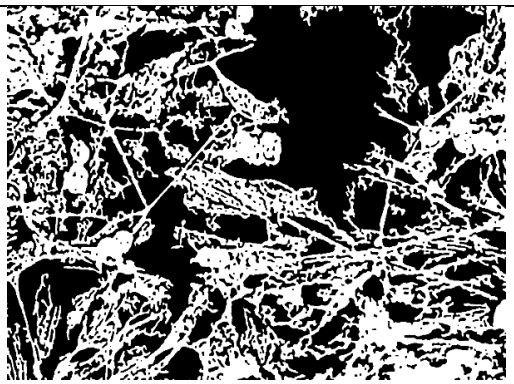
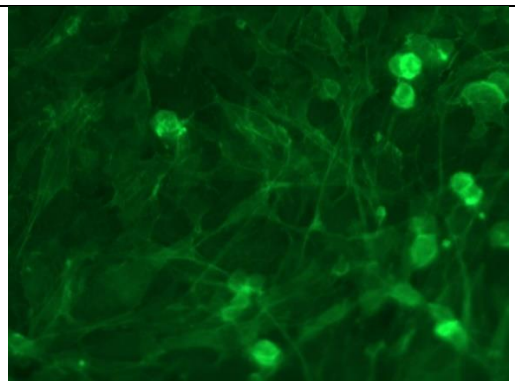
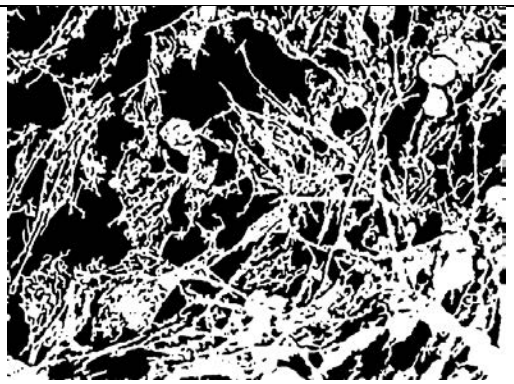
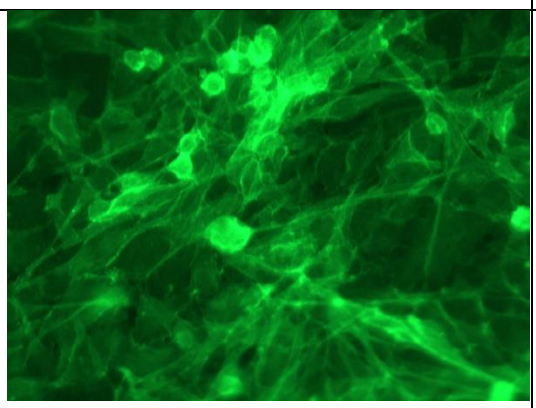
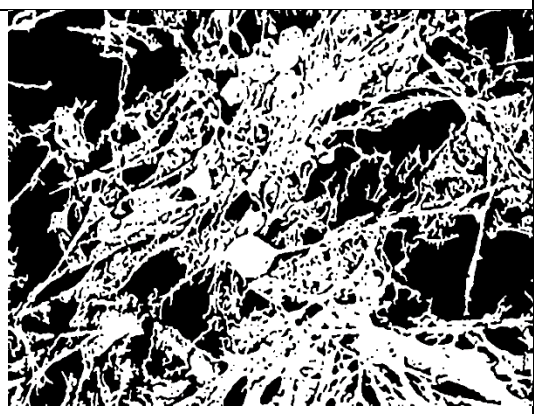
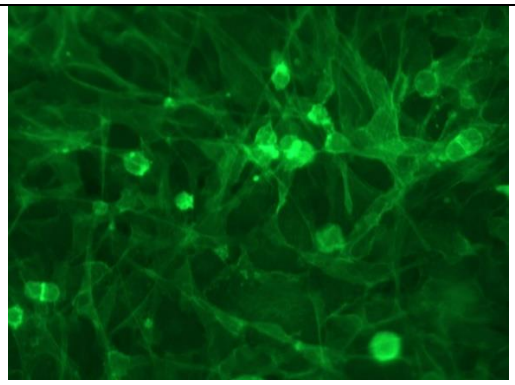
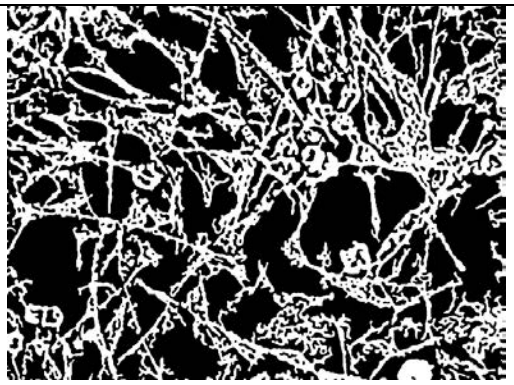
results in actin remodeling, which could lead to treatments for bovine mastitis and other inflammatory diseases in the future.

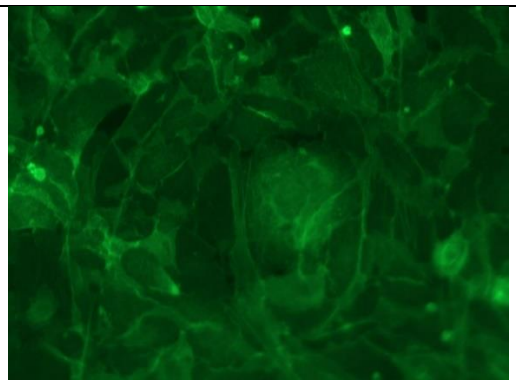
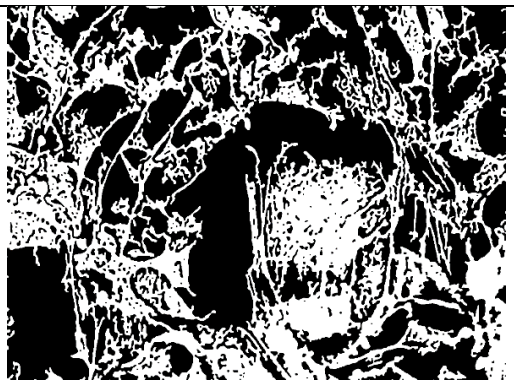
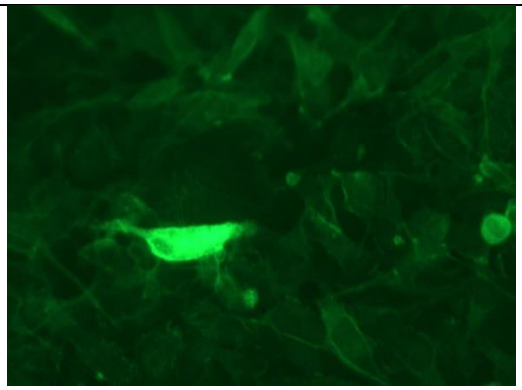
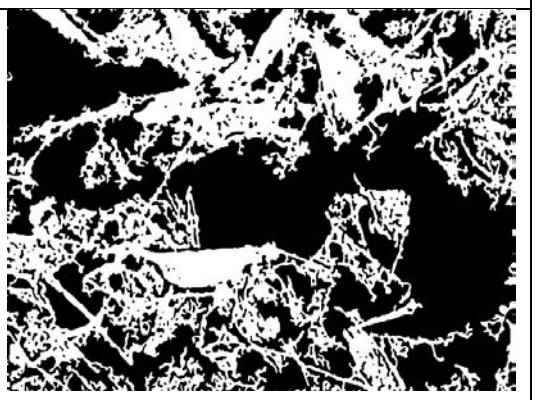
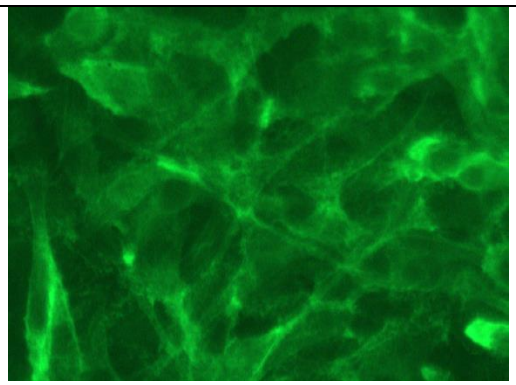
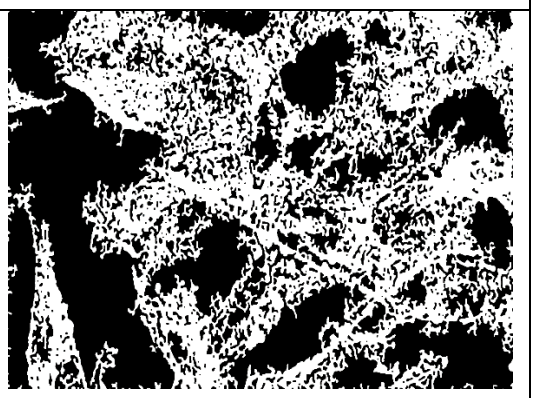
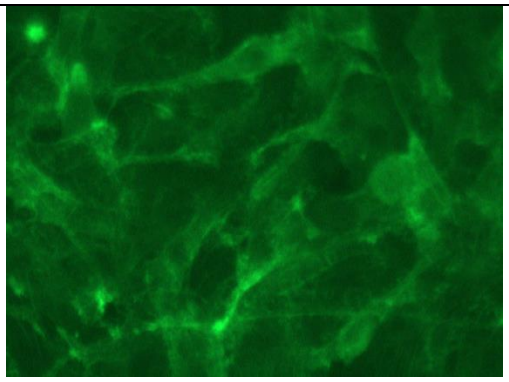
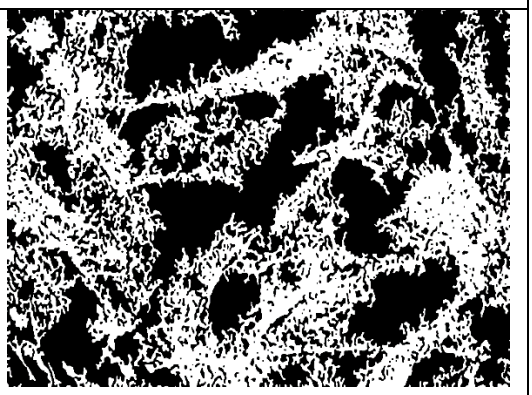
The potential impact of this project is finding possible treatments to bovine mastitis and inflammatory diseases or an increased number of treatment options. Finding treatments for bovine mastitis could impact developing countries by increasing milk production, an important resource. Treatments for inflammation can make a big difference in the medical field, as many disease states are compounded by inflammation. Better treatments for inflammation can help relieve some of these symptoms and improve quality of life for bovine as well as humans when considering conditions associated with extracellular ATP.

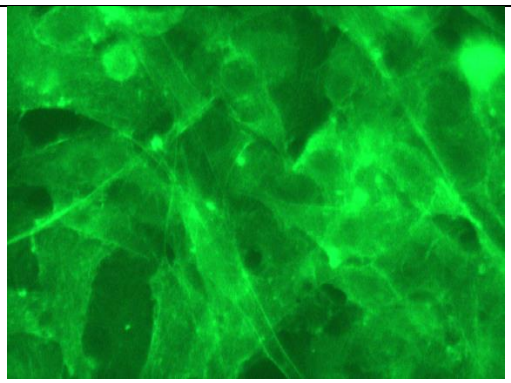
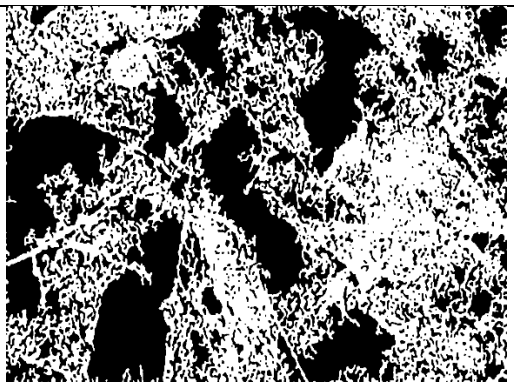
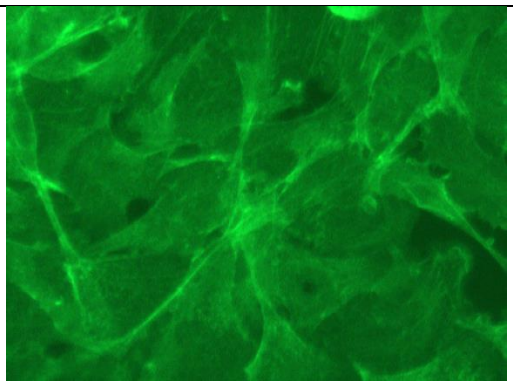
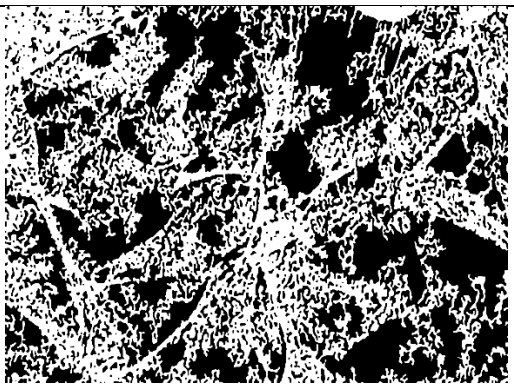
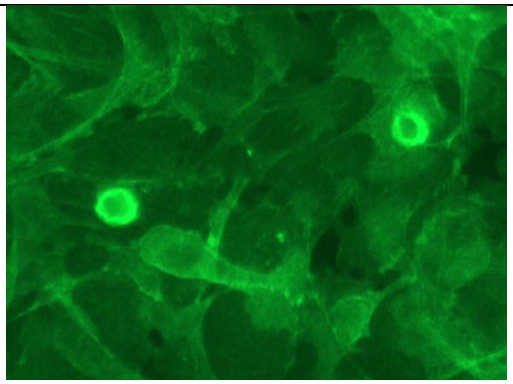
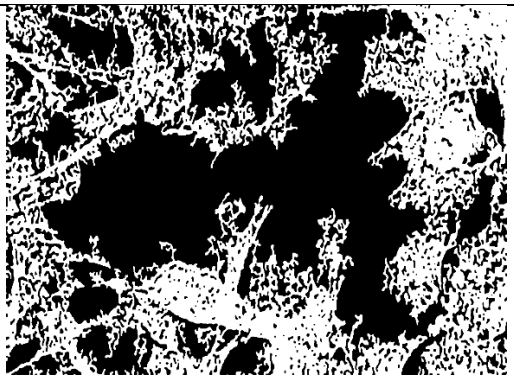
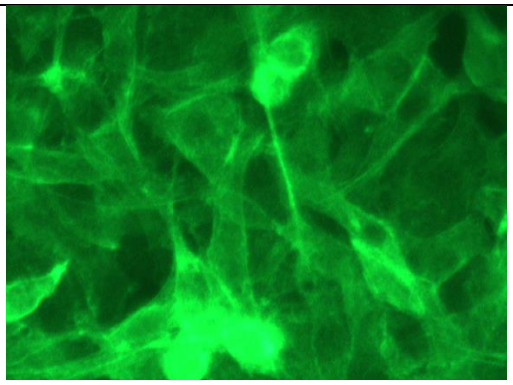
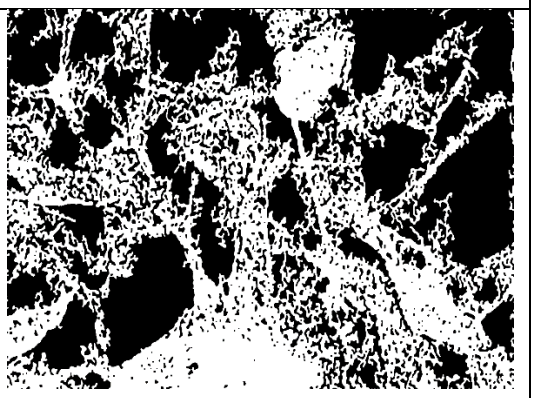
Appendix

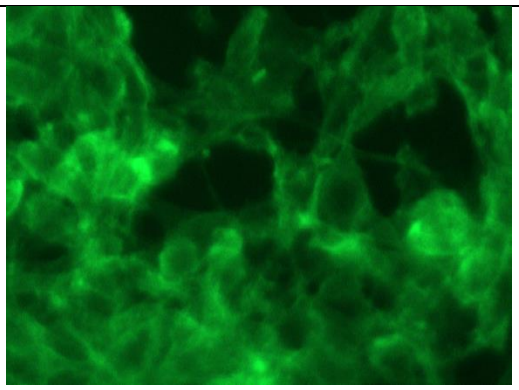
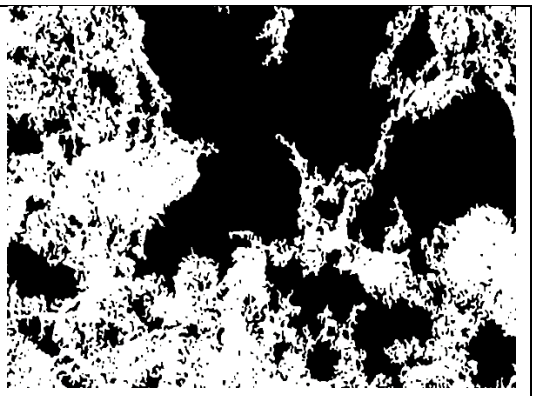
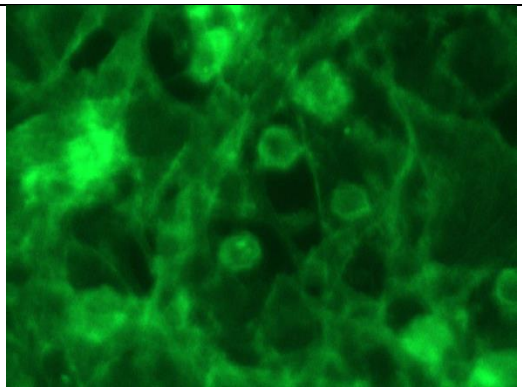
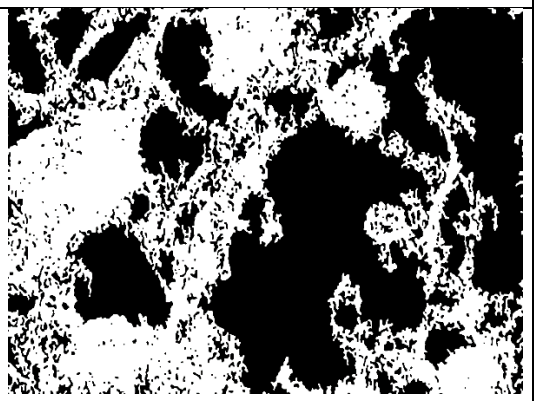
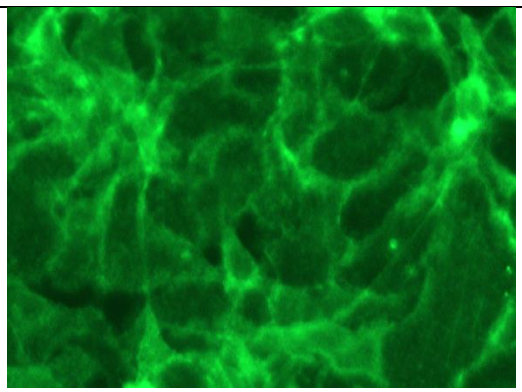
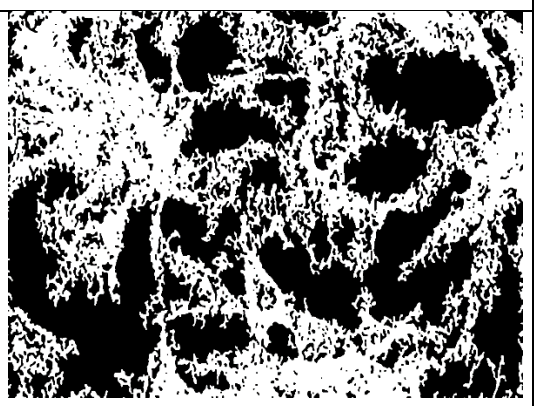
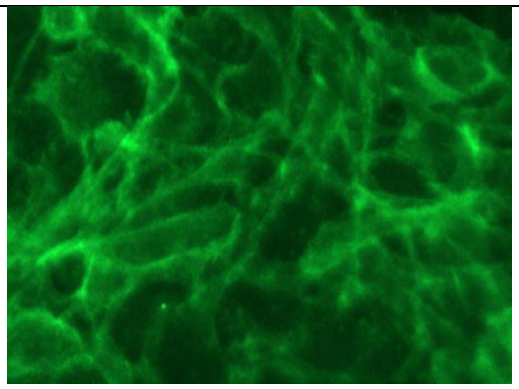
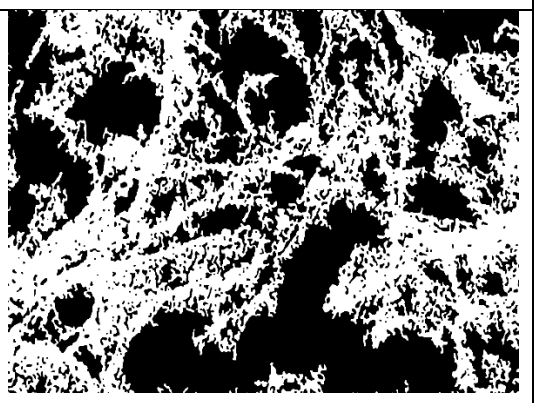
Table 1: Fluorescent Images and Corresponding Segmented Images

Date	Treatment	Original Image	Segmented Image
4/28/17	No Treatment 1		
4/28/17	No Treatment 2		
4/28/17	No Treatment 3		

4/28/17	5 min ATP 1		
4/28/17	5 min ATP 2		
4/28/17	15 min ATP 1		
4/28/17	15 min ATP 2		

4/28/17	30 min ATP 1		
4/28/17	30 min ATP 2		
6/2/17	5 min ATP 1		
6/2/17	5 min ATP 2		

6/2/17	15 min ATP 1		
6/2/17	15 min ATP 2		
6/2/17	30 min ATP 1		
6/2/17	30 min ATP 2		

6/7/17	5 min ATP 1		
6/7/17	5 min ATP 2		
6/7/17	15 min ATP 1		
6/7/17	15 min ATP 2		

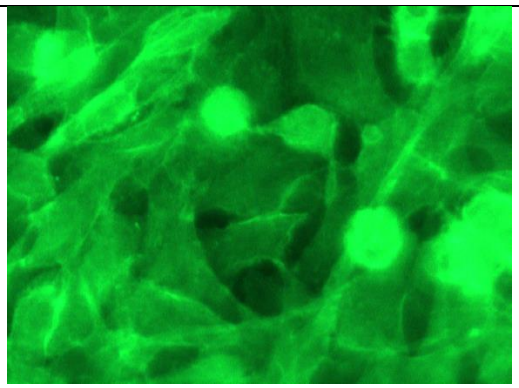
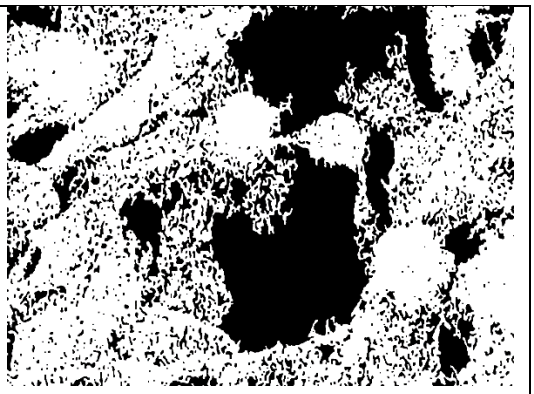
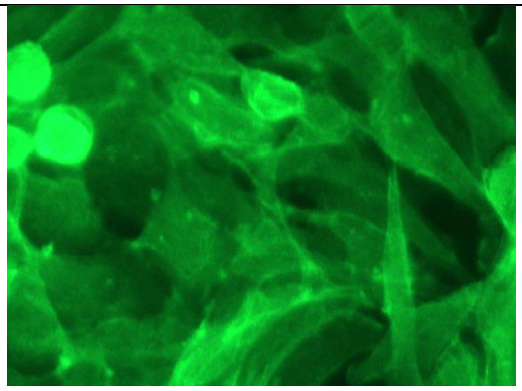
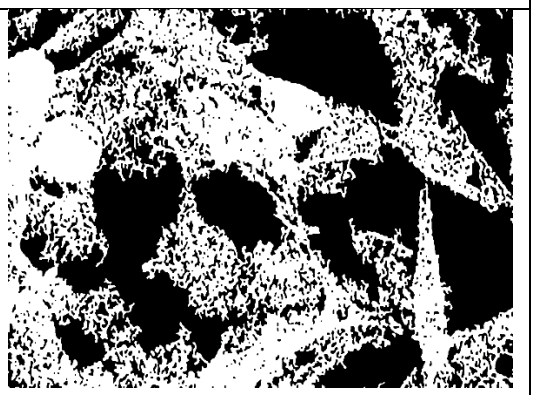
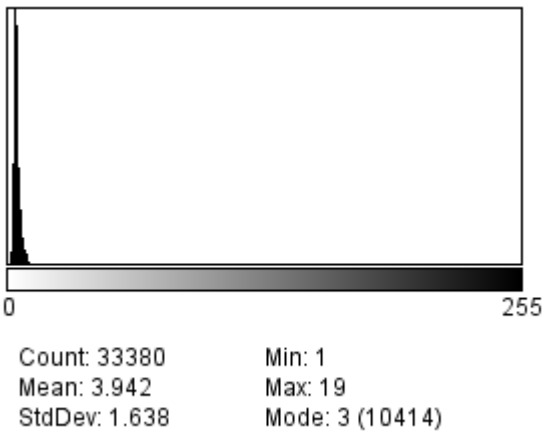
6/7/17	30 min ATP 1		
6/7/17	30 min ATP 2		

Table 2: Fiber Diameter Histograms

Date	**Treatment**	**Average Fiber Diameter (µm)**	**Fiber Diameter Histogram**
4/28/17	No Treatment 1	3.942	

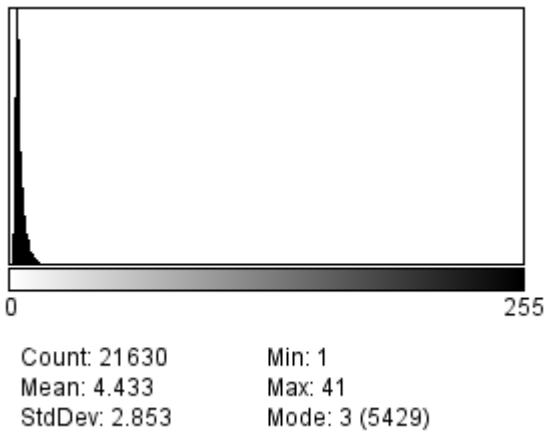
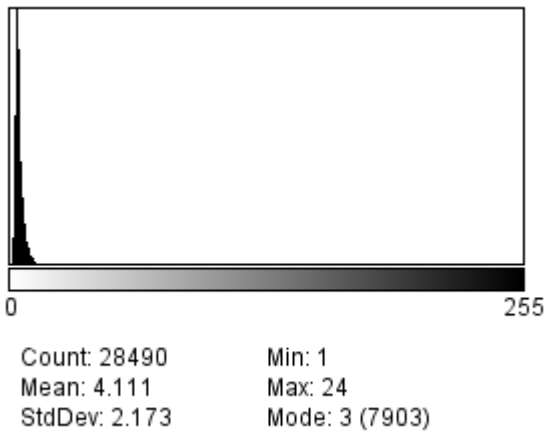
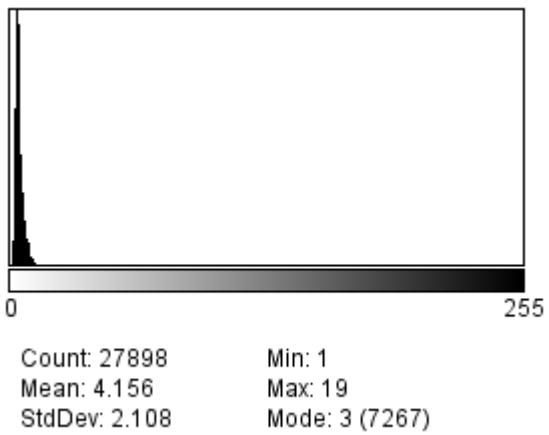
4/28/17	No Treatment 2	3.898	
4/28/17	No Treatment 3	4.208	
4/28/17	5 min ATP 1	4.056	

4/28/17	5 min ATP 2	3.925	
4/28/17	15 min ATP 1	4.330	
4/28/17	15 min ATP 2	4.284	

4/28/17	30 min ATP 1	4.085	
4/28/17	30 min ATP 2	4.156	
6/2/17	5 min ATP 1	3.846	

6/2/17	5 min ATP 2	3.713	
6/2/17	15 min ATP 1	3.682	
6/2/17	15 min ATP 2	3.530	

6/2/17	30 min ATP 1	3.768	
6/2/17	30 min ATP 2	3.900	
6/7/17	5 min ATP 1	4.413	

6/7/17	5 min ATP 2	4.433	
6/7/17	15 min ATP 1	4.111	
6/7/17	15 min ATP 2	4.156	

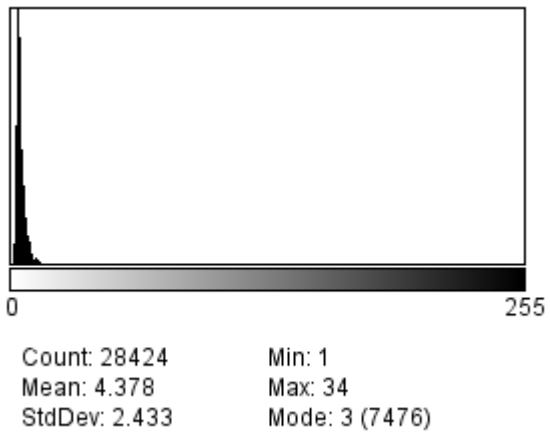
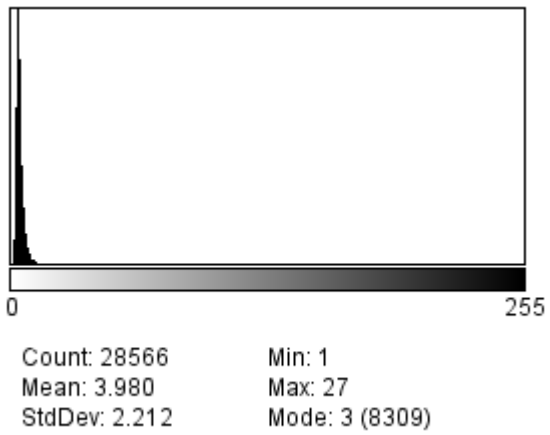
6/7/17	30 min ATP 1	4.378	
6/7/17	30 min ATP 2	3.980	

Table 3: Percentage of Actin Present

Date	**Treatment**	**Percent Black Pixels**
4/28/17	No Treatment 1	0.4107
4/28/17	No Treatment 2	0.3562
4/28/17	No Treatment 3	0.4710
4/28/17	5 min ATP 1	0.4209
4/28/17	5 min ATP 2	0.4529
4/28/17	15 min ATP 1	0.5617

4/28/17	15 min ATP 2	0.5164
4/28/17	15 min ATP 3	0.3687
4/28/17	30 min ATP 1	0.4187
4/28/17	30 min ATP 2	0.4057
6/2/17	5 min ATP 1	0.4582
6/2/17	5 min ATP 2	0.4430
6/2/17	15 min ATP 1	0.4894
6/2/17	15 min ATP 2	0.4695
6/2/17	30 min ATP 1	0.4159
6/2/17	30 min ATP 2	0.4664
6/7/17	5 min ATP 1	0.4392
6/7/17	5 min ATP 2	0.4499
6/7/17	15 min ATP 1	0.4626
6/7/17	15 min ATP 2	0.4695
6/7/17	30 min ATP 1	0.6037
6/7/17	30 min ATP 2	0.4756

Note: Data given in percent black pixels was converted to percent white pixels before making the graph.

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