Effects of Cytochalasin D on CHO Cell Morphology and Virus Factory Formation in L929 Cells

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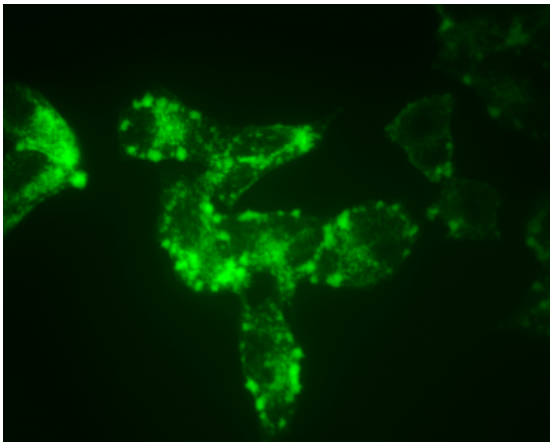
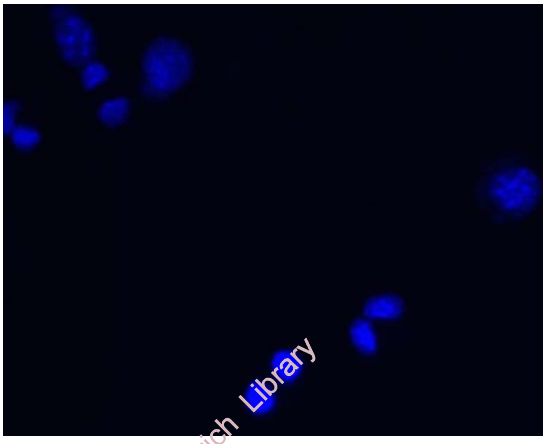
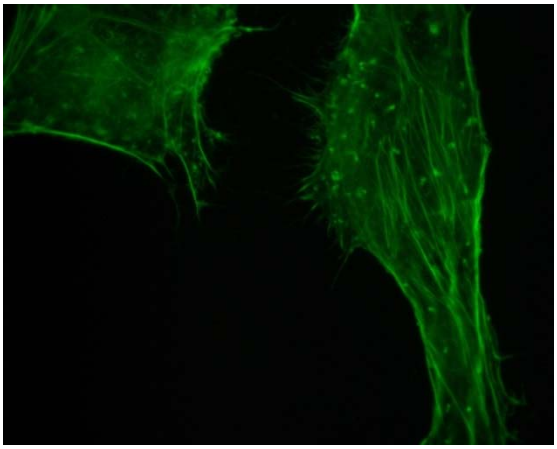
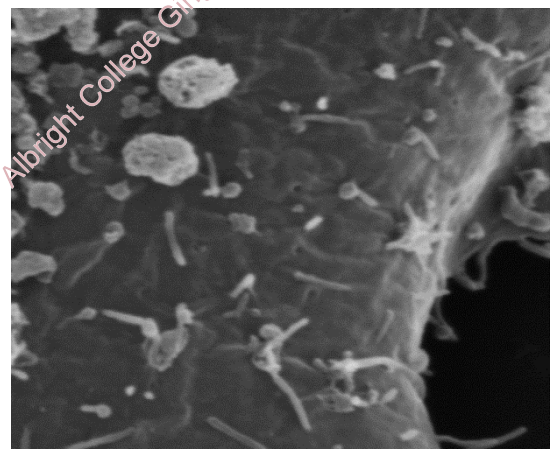








Abstract:

Actin networks can be found in eukaryotes in their cell cortex, just under their plasma membrane. Lamellipodia and Filopodia are thin protrusions that can be extended from the cell cortex. Both of these protrusions are involved in creating an anchorage system for cells. Movement and growth are driven by actin polymerization which pushes the plasma membrane forward and forms new regions of actin cortex. Cytochalasin D (CD) is a cell permeable toxin that binds to the growing end of actin filaments disrupting the actin networks and preventing polymerization. In a previous study conducted by Jenna Deibert, Class of 2013, Chinese hamster ovary cells were grown for 24 hours then treated with concentrations of varying concentrations of CD ranging from 0-10 μg/mL for 24 hours. The effects of CD on actin filaments were viewed with scanning electron and fluorescence microscopy. This experiment was repeated with the addition of a cell rinsing and recovery component. Because pox virus replication is an actin-driven process, the effects of virus replication were also studied using CD. Cells were recovered and harvested for both fluorescence and scanning microscopy at 24 hours, 48 hours, and 72 hours to observe the restoration of actin networks along with the effects of virus factory production.

Introduction:

Actin networks provide strength, support, and aid in cellular movement and attachment and can be found in eukaryotes in their cell cortex. Lamellipodia are the projections that are found in the leading edge of the cell and contain a dense meshwork of actin. Filopodia are thin protrusions that are made of loose bundles of actin and can be extended from the cell cortex. Both of these protrusions are involved in creating an anchorage system for cells (Figure 1).

Movement and growth are driven by actin polymerization which pushes the plasma membrane forward and forms new regions of actin cortex (Alberts *et al*, 2010). Actin filaments are essential for movement, maintaining structure, and cell division. The state of actin filaments strongly affects the structural and mechanical properties of cells and tissues. By studying the role of actin in the cell, much insight can be gained about cellular processes.

Actin filaments, also known as F-actin is made up of individual globular actin (g-actin) polypeptides which become polymerized. Actin filaments grow with the addition of g-actin at either the plus or minus end with growth rate faster the plus end, a process known as treadmilling. When the actin monomers bind to ATP, it is hydrolyzed to ADP, allowing polymerization to occur. Small proteins bind to g-actin in the cytosol preventing them from attaching to the ends of the actin filaments, regulating actin polymerization. Within in the cell, assembly and disassembly of actin filaments are regulated by actin-binding proteins (Alberts *et al*., 2010). Actin polymerization is also reversible. Filaments can depolymerize by the dissociation of actin subunits, allowing actin filaments to be broken down when necessary.

In order to study the disruption and reversibility of the actin networks, a toxin was added and cells were allowed to recover. Cytochalasin D (CD) is a cell permeable toxin that binds to the growing end of actin filaments which prevents polymerization and disrupts the actin filaments, thus changing the cell shape. CD induces an increase in the initial rate of polymerization with a dramatic decrease in the final extent of the reaction and increases the concentration of monomeric actin, both of which depend on CD induced formation of dimers. (Goddette and Frieden, 1986).

Cytochalasins have greatly strengthened scientific knowledge about actin filaments in biological processes (Alberts *et al.*, 2010). Visible changes in cells treated with CD were withdrawal of microvilli, loss of movement, cytoplasmic contraction resulting in diminished cell area, and aggregation of protrusions of blunt, knobby projections of endoplasm at the cell surface known as zeiosis (Miranda *et al.*, 1974a). The cell cortex also experiences changes after exposure to CD. Instead of being evenly arranged, large aggregates of filaments occupy the cortex (Miranda *et al.*, 1974b). Normally, actin filaments form end to side associations with other actin filaments as well as with microtubules and intermediate filaments, making the cytoskeleton highly interconnected. When CD is introduced, this network is disrupted. The components remain, but distribution becomes irregular and non-uniform. Some areas are completely devoid of actin filaments while others have large bunches. These large bunches consist primarily of actin filaments, but this reorganization of actin filaments also changes microtubule and intermediate filament distribution. The discontinuous filaments appear to be broken, cut, and disconnected from other filaments. This suggests that CD disrupts network organization by removing some of its component filaments and releasing them into the cytoplasm (Schliwa, 1982).

Studies have shown that virus infection utilizes the actin cytoskeleton. The virus used in this study is the Ectromelia virus (ECTV), which is an orthopoxvirus and whose natural host is the mouse. Poxviruses are viruses that carry DNA and are unique to DNA viruses because they replicate in the cytoplasm rather than the nucleus (Schramm and Locker, 2005). Viruses create cytoplasmic "mini-nuclei," also known as virus factories, which are used for virus replication. Actin networks are seen to play important roles in the life cycle of many viruses. It has been shown that viruses reconfigure and reorganize the actin networks throughout all of their cycle,

from entry into a cell to assembly of virus particles. When virions engage with the cell surface, the cell membrane must be penetrated. The physical barrier imposed by the actin network must be overcome which involves remodeling of the actin cytoskeleton. Viral proteins located on the surface will bind to cellular receptors which are associated with actin filaments, including filopodia (Figure 2). These interactions stimulate movement of actin networks and lead to fusion or endocytosis. This process is known as virus surfing. During surfing, the virus is coupled with the cytosolic domain of its receptor to an actin filament inside the filopodium. This filament may then be pulled towards the base of the filopodium by myosin II which itself is anchored to the actin cortex .The actin filaments continuously polymerize at the tip of the filopodium to maintain its length. This surfing may provide transportation to a region of the plasma membrane where virus internalization is possible (Radtke *et al.,* 2006). Virion surfing has been known to be sensitive to cytochalasin D. Actin also plays a role in the final assembly and budding of infectious virions at the cell surface. During the late stages of its replicative cycle, viruses use actin filaments to spread directly from cell to cell through the formation of actin tails. Blebs also form on the surface of the cell through a process known as budding (Boratynska *et al*., 2010). In previous studies, disrupting actin filaments with cytochalasin D dramatically blocked virus budding in a mouse mammary tumor virus (Taylor *et al.*, 2011).

Previous studies in our lab have focused on the disruption on actin networks for multiple cell types. Focusing on the reversibility and restoration of the actin network has not been previously studied in our lab in conjunction with CD. We treated CHO cells with concentrations of CD ranging from 0 – 10 µg/mL for 24 hours and then washed the cells to remove the drug and allow the cells to recover. We furthered the study by infecting L929 cells with ECTV poxvirus and CD to see the affects CD would have on virus replication. Based on previous studies, we

hypothesized that: (1) CD will bind to actin filaments in CHO cells preventing elongation of actin and polymerization resulting in visual differences in cell shape and appearance, and full recovery will be seen after 24 hours of removal of cytochalasin D and (2) cytochalasin D will inhibit virus factory formation in L929 cells when infected with Ectromelia virus.

Materials and Methods:

Cell Maintenance: CHO cells, cell line CHO-K1 ATCC CCC-61, and L929 cells were maintained in a T-25 flask containing Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), and Penicillin-Streptomyocin antibiotics (PS) (100 µL/mL). Cells were grown in a CO_2 incubator at 37°C and 10% CO_2. Confluency, morphology, and contamination of cells were analyzed under an inverted phase microscope. To ensure a sterile environment, all work with live cells was performed in a laminar flow hood and all materials used were in sterile packaging or sterilized by autoclaving before use.

Subculturing: Cell cultures were subcultured periodically to maintain the cell line. To subculture, cells were first rinsed three times with 5 mL of a room temperature isotonic phosphate buffered saline (PBS) free of calcium and magnesium ions. This was performed to remove the bovine serum in the medium, which contains a natural trypsin inhibitor and must be removed to allow trypsin to work. PBS also dilutes the calcium and magnesium ions already present which helps cells attach to a substrate. Next, 1 mL of crude trypsin, preparation of 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in HBSS that was pre-warmed from its frozen state in a 37°C water bath, was added to the flask. The cells were then placed into a 37°C incubator for one minute. This process allows the proteolytic enzyme trypsin to remove cells from the surface of the flask by breaking intercellular and cell-to-substrate connections.

2mL of DMEM medium containing 10%FBS and PS, which was pre-warmed from its refrigerated state in a 37°C water bath, was added to the flask to inactive the trypsin; FBS contains a natural trypsin inhibitor.

Cell counting: To count the cells, 0.1 mL of cells was removed from the flask and added to a microcentrifuge tube along with varying amounts of 0.04% trypan blue in HBSS as a dilution factor based on the density of cells. Trypan blue is used to test cell vitality; viable cells will exclude the dye whereas dead cells will stain blue due to membrane permeability. The microcentrifuge tube was vortexed for a couple seconds and cells were immediately loaded into one chamber of a hemocytometer. The cells were vortexted again and then loaded into the other chamber of the hemocytometer. The cells were examined using a light microscope under 100x magnification, with a field of view of one square millimeter (mm). Five of the nine one square mm squares from each of the chambers were counted to determine the number of cells per mm^3. Cells that touched the left, upper, and middle of the three boundary lines were included in counting. In order to obtain the number of cells per mL, the number of cells counted was multiplied by the dilution factor and 1000. Cell count was used to determine how to seed 1.0 x 10^5 cells in 5mL medium when subculturing (see calculation 1).

Scanning electron microscopy: To prepare cells for visualizing, cells were counted using the same procedure to determine how to seed 1.0 x 10^5 cells in 12mL medium (see calculations 1-2). Three sterile 5mm x 5mm silicon wafers were placed in each well of a six well plate. Silicon wafers provide a conductive surface for scanning electron microscopy. Each well was seeded with 2 mL and placed in the CO_2 incubator to allow cells to grow. After 24 hours, cytochalasin D (from a 5mg/mL stock solution) in dimethyl sulfoxide (DMSO) was added to the cells in varying concentrations of 0-10 µg/mL (Table 1) using micropipettes. DMSO was used as

a control because it is an organic compound that dissolves CD and does not cause any reaction in the cells at low concentrations. The plate was then placed back in the CO_2 incubator for another 24 hours. After 24 hours, one wafer from each well was harvested and fixed, treated with osmium, and dehydrated. The wells were rinsed three times with 2 mL of PBS and 2mL of DMEM medium with 10%FBS and PS was added and the cells were placed back in the CO_2 incubator. This removed all the CD and allowed the cells to recover. To fix cells, cells were rinsed three times with 2mL of PBS. 2 mL of Karnovsky's fixative (2% paraformaldehyde, 2.5% gluataraldehyde, 0.1 M buffer) with 5% sucrose was added to the cells for one hour in a fume hood because cells were no longer viable. Karnovsky's fixative with 5% sucrose was made with 13 mL of 16% paraformaldehyde solution, 5 mL of 50% gluataraldehyde EM grade, 50 mL of 0.2 sodium phosphate buffer, 32 mL deionized water, and 5 grams sucrose. After one hour incubation, cells were postfixed with osmium tetraoxide to make them conductive. Equal parts of 4% osmium tetraoxide in premade ampule and 0.2 M pH 7.4 phosphate buffer were added and cells were incubated in the hood for one hour. Cells were than dehydrated using an ethanol series of 30%, 50%, 75%, and 100% ethanol for five minutes each. After one change of 100% ethanol, samples underwent critical point drying. Critical point drying allows the sample to dry with no surface tension forces, which would cause the tissues to shrivel and be distorted. By using controlled temperature and pressure, surface structures of the cells were maintained. Ethanol is replaced with liquid CO_2 which is then turned into gaseous CO_2 with increasing temperature. Each silicon wafer was then sputter coated with gold, allowing them to be conductive. The surface topography of cells was then looked at using a scanning electron microscope (SEM). Two random spots were chosen from each wafer and viewed at 500x magnification. From each 500x spot, three random cells were chosen using a clear sheet held over the screen in which five

spots were randomly chosen (two extra spots were allotted in case there were no cells at one of the chosen spots). The cells were viewed at either 2070x, 2030x, or 2010x magnification. Magnifications varied between viewing sessions due to the inability to manually input magnification when zooming in on an already magnified image. All images were saved to iTable in a 512 pixel x 512 pixel TIF format. After 24 hours, another wafer was harvested by being fixed, treated with osmium, dehydrated and viewed under the scanning electron microscope using the same procedure. The last wafer was placed back in the CO_2 incubator for 24 hours. After another 24 hours, the last wafer was harvested by being fixed, treated with osmium, dehydrated and viewed under the scanning electron microscope using the same procedure.

Fluorescence microscopy: The cells were also viewed under fluorescent microscopy to view the actin filaments. The same procedure was used for cell preparation during subculturing. Cell count was used to determine how to seed 1.0×10^5 cells in 12 mL of DMEM medium (see calculation 3-4). Two sterile glass coverslips were placed in each well of a six-well plate. Each well was seeded with 2mL of DMEM medium and placed back in the CO_2 incubator to allow the cells to grow. After 24 hours, CD in DMSO was added to cells in varying concentrations of 0-10μg/mL (Table 1) using micropipettes. The plates were then placed back in the CO_2 incubator for another 24 hours. After this incubation period, one coverslip was removed and harvested by being rinsed twice with 2mL of PBS while the medium was removed from the other coverslip, rinsed three times with PBS, and 2mL of fresh DMEM medium was added. The six-well plate was then placed back in the CO_2 incubator. 2 mL of %10 buffered formalin phosphate was added to the harvested coverslip and the plate was incubated for ten minutes at room temperature. The fixative was then removed and coverslips were rinsed three times with 2mL of PBS. Previously, a 50mL beaker and Coplin jar containing acetone were placed into the freezer (-20°C).

Coverslips were quickly dipped ten times in acetone and then placed in the Coplin jar. The coplin jar was then placed in the freezer for ten minutes. Acetone allows the cells to permeabilize. The coverslips were then removed and cells were rehydrated by the coverslips sequentially in two beaker of PBS. The coverslips were then placed in a 35 mm dish with 2mL of PBS for ten minutes, with cells facing up. A moist chamber was prepared by placing a piece of Whatman #1 filter paper into a glass petri dish and wetting it with deionized water. Coverslips were placed in the chamber on inverted microfuge caps. 185 µL of BODIPY FL phallacidin in PBS (20 units/mL) + 1% BSA (bovine serum albumin) was added to each coverslips and coverslips were incubated for 30 minutes in the dark. BODIPY FL phallacidin is a fluorescent dye that binds to actin filaments and allows these filaments to be viewed green under the fluorescent microscope. After incubation, coverslips were dipped sequentially in PBS and DI water five times each. Coverslips were placed on end until completely dried and mounted on a microscope slide using mounting medium, cytoseal60. After allowing the mounting medium to dry for 24 hours, cells were viewed under the fluorescence microscope using the filter set for fluorescein under 10x and 40x magnification. After 48 hours of recovery, the last coverslip was harvested and prepared for fluorescent microscopy using the same procedure. To prepare slides for viral infection, the same procedure as above was followed with the following exceptions: Cells were treated with plaque forming units per cell of virus (calculation 5) alone and simultaneously with 5µg/mL of CD. Cells were also mounted onto slides using a mounting medium containing DAPI. DAPI binds nonspecifically to DNA, allowing the nucleus and any virus factories to be viewed blue in color with the fluorescence microscope.

RESULTS:

When the CHO cells were treated with cytochalasin D, actin networks were visibly disrupted in scanning electron microscopy (Table 2) and fluorescence microscopy (Table 6). However, when cytochalasin D was removed from the cells, recovery was seen after 48 hours. The control cells that were viewed with SEM are very flat and display the typical fibroblast-like morphology of CHO cells. The disruption of the actin networks can be seen at the lowest concentration of 1 μg/mL. Cells shrink in size and become more rounded as the concentrations increase. The morphology of the cells from 1μg/mL to 10 μg/mL all have the general same appearance with increased filopodia protusions. The higher the concentration of CD, the smaller and more rounded the cell and the longer and more disordered the filopodia protrusions (Table 2). Under fluorescence microscopy, the control cells (no CD treatment) display a highly organized cell cortex with actin filaments evenly distributed throughout the cell (Table 6). When the drug is introduced into the cells, it is seen that the actin networks begin to bundle together even at the lowest concentrations and the appearance seems to become more rounded (Table 6). As the concentrations of CD increase, the actin filaments become more bundled together (Table 6).

When the cytochalasin D was removed from the cells and incubated for an additional 24 hours, some recovery was seen using scanning electron microscopy (Table 3). Recovery occurred faster in the lower concentrations of 1 μg/mL than in the 10 μg/mL (Table 3). After 48 hours, full recovery of actin filaments and cell morphology was seen in all concentrations (Table 4). After 72 hours, recovery was seen at all concentrations except for 1 μg/mL due to contamination (Table 5). However, based on the trends, it is assumed that 1μg/mL would have

had a full recovery. Although cells were recovered, the shape of the cell seems to be wider when compared to unaffected, control cells. Using fluorescent microscopy, CHO cells that were recovered for 48 hours were seen to have a recovered, well organized cell cortex of actin filaments at all concentrations (Table 7).

When L929 cells were infected with a multiplicity of infection of 5 with ECTV (calculation 5), virus factories were seen using fluorescence microscopy (Table 8) while actin tails were seen in both fluorescence microscopy and SEM (Table 9). Actin tails can be viewed when cells were treated with virus for 24 hours (Table 9). The formations of blebs were also seen on the surface of cells infected with ECTV (Table 10). Virus factories can be seen in the cytoplasm and were seen in 100% of cells treated with virus for 24 hours without CD. However, when 5 μg/mL CD was added with the ECTV, there was a dramatic decrease in the amount of virus factories produced (Table 8). Out of 50 cells, 100% of cells treated with virus contained virus factories whereas only 46% of infected cells treated CD contained virus factories (Figure 4).

DISCUSSION:

The reversibility of actin networks was seen throughout recovery of treatment from cytochalasin D after 48 hours (Table 4 and 6). The disruption of actin networks in cells treated with CD was again seen using both electron microscopy (Tables 2) and fluorescent microscopy (Table 6). SEM and fluorescence microscopy images displayed cytoplasmic contraction resulting in diminished cell size and aggregation of protrusions of blunt, knobby projections of endoplasm at the cell surface (Miranda *et al*., 1974). With the onset of contraction microvilli are withdrawn from the cell surface and blunt processes are protruded along the cell periphery. These processes

enlarge and form knobs which join into larger groups. These often mass near the cell apex forming one or two large aggregates. The term used to describe the protrusion of these processes is zeiosis (Godman *et al.*, 1975). At CD concentrations of 2.5 µg/mL and above, filopodia protrusions are much longer and more disordered than at lower concentrations. As concentration increased, bundles of actin filaments began to appear throughout the cell. The bundles of actin filaments became larger and moved more towards the center of the cell (Table 6). Fluorescence microscopy also displayed changes in the cell cortex. Cells maintained a fibroblast like shape, but actin filaments became less visible surrounding the cell as concentration increased (Table 6). After disruption of the actin network within the cell cortex, the components remain, but distribution becomes irregular and non-uniform. The discontinuous filaments appear to be broken, cut, and disconnected from other filaments. This suggests that CD disrupts network organization by removing some of its component filaments and releasing them into the cytoplasm (Schliwa, 1982). It has been shown that the cell cortex experiences changes after exposure to CD. Instead of being evenly arranged, large aggregates of microfilaments occupy the cortex (Miranda *et al.*, 1974b).These observations were all seen in a study conducted by Mirdanda *et al*. and by Jenna Diebert.

However, when the cells were washed and the drug was removed, actin networks were recovered as well as the cell morphology (Tables 3-5). In the 24 hour recovery period, cells that had been exposed at lower concentrations to cytochalasin D were seen to recover while the cells exposed to 10 µg/mL did not fully recover until 48 hours. At 48 hour recovery, full recovery of actin networks and morphology was seen at all concentrations. However, recovery is more prolonged with higher concentrations of CD. Large, well organized longitudinal bundles of thin microfilaments occupy much of the cortex in untreated cells. When cells recovered, the cell

cortex was not as organized and seemed to be wider in size than the original untreated cells. Although the cortex is not as organized compared to untreated cells, both filopodia and lamellipodia protrusions were recovered and participated in creating an anchorage system for cells. This demonstrates the reversibility of actin polymerization, which may be beneficial in further studies with using cytochalasin D to inhibit virus shedding.

The scope of this research was furthered by studying the effects of cytochalasin D on virus replication, specifically, ECTV, a poxvirus. Poxviruses use actin filaments and other components of the cytoskeleton to facilitate entry into a cell, assembly of virion particles, and to facilitate infections of neighboring cells. Before viruses enter the cell, they interact with the actin cytoskeleton through virus surfing by binding to filopodia. Treatment with CD has resulted in undirected wandering of virions on the cell surface leading to a reduction in viral infectivity (Taylor *et al.*, 2011). When cells were treated with both CD and ECTV, there was a significant decrease in the amount of virus factories, from 100% to only 46%. This suggests that when actin networks are disrupted, viruses cannot enter a cell and infect it. Some viruses may enter but not be able to traffic to the appropriate place in the cell to being with replication cycle.

The formation of actin tails and the process of budding are classic signs of infection of poxviruses and were seen with treatment of ECTV. Both of these processes use the cells actin networks. When poxviruses are ready to infect other cells, they need to move past the actin network and use the cytoskeleton in order to spread. Viruses are known to produce blebs on the surface through a process known as budding. Although we were not able to see the effects that CD had on budding, previous studies have indicated that treatment with cytochalasin D blocked virus budding by 80% (Maldarelli *et al*., 1987). The formations of actin tails are essential for inter-cell propagation of the infection and were seen in all cell types observed (L929 and BSC-

1). The formation of these tails are propagated by the actin cytoskeleton. Further research will be performed to see the effects cytochalasin D has on the formation of actin tails although it is proposed that there will be a significant reduction in tails due to the disruption of actin.

ACKNOWLDEGMENTS:

I would like to thank Dr. Andrew Samuelsen, for not only being my faculty advisor, but for his continued support and guidance throughout my research. I would also like to thank Dr. Adam Hersperger for his contributions by furthering my research to encompass viruses, as well as his continued encouragement. Furthermore, I would like to thank Dr. Richard Heller for his help and guidance with the scanning electron microscope. Lastly, I would like to thank my college reader Dr. Lisa Bellantoni for her motivation.

Appendix

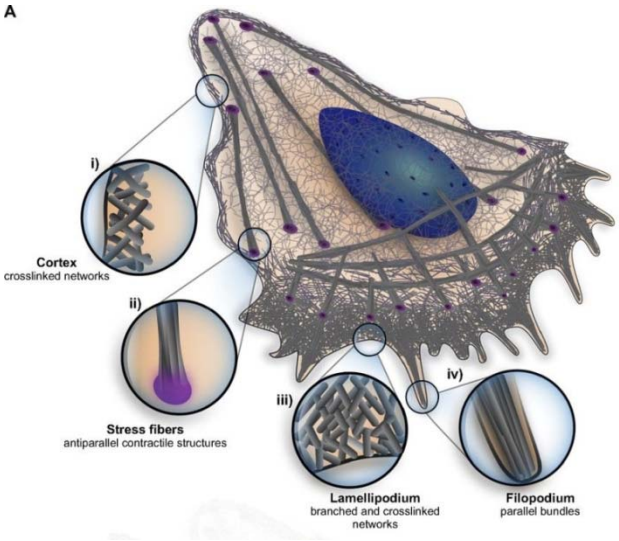


Figure 1: Actin filaments, Lamellipodium and Filopdidum, distribution in cell and tissues; Used by permission from physrev.physiology.org; American Physiological Society

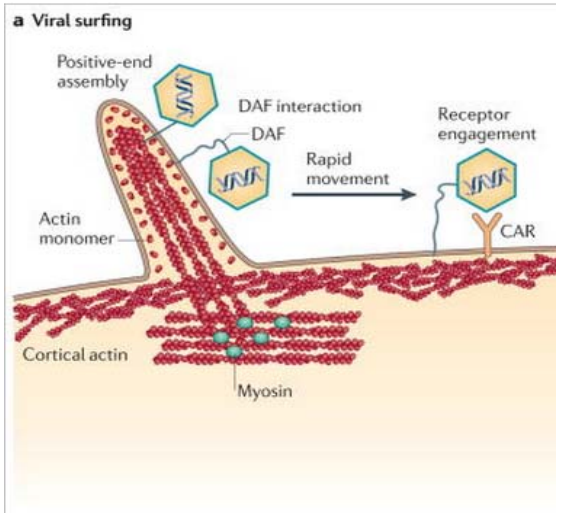


Figure 2: Viral surfacing is facilitated by actin filaments; used by permission from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3229036/

CD concentration (µg/mL)	DMSO added (µL)	CD added (µL)
0 (control)	4.0	0
1.0	3.8	0.2
2.0	3.6	0.4
2.5	3.5	0.5
5.0	3.0	1.0
10.0	2.0	2.0

Table 1: Various µL of DMSO and CD used in experiment

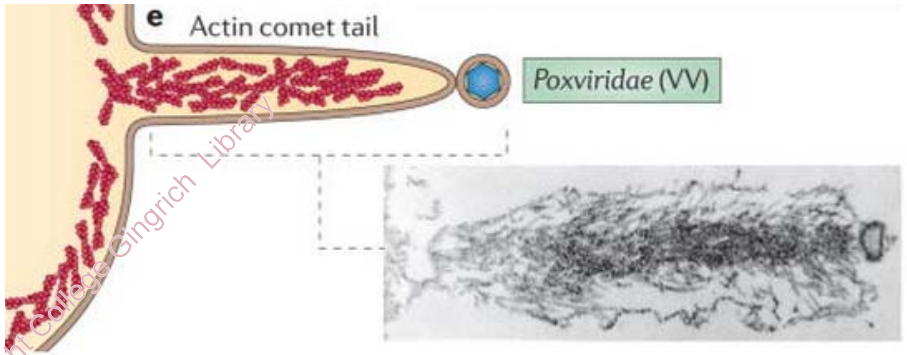


Figure 3: Actin filaments are used to spread directly from cell to cell through the formation of actin tail; used by permission from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3229036/

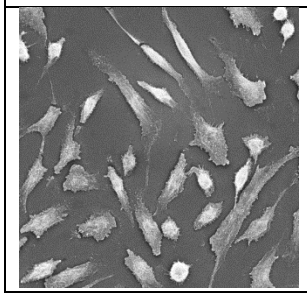
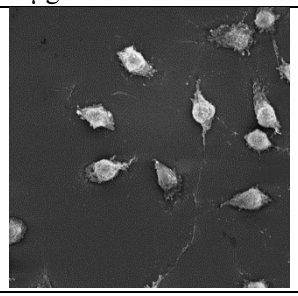
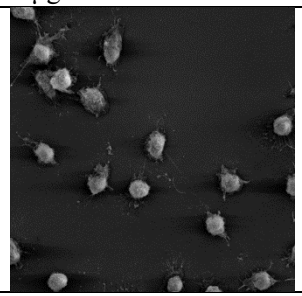
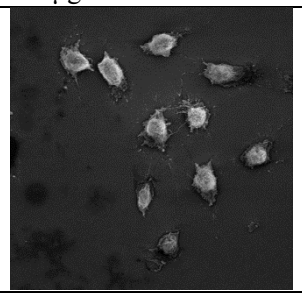
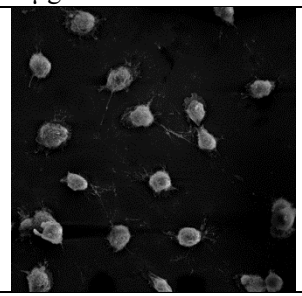
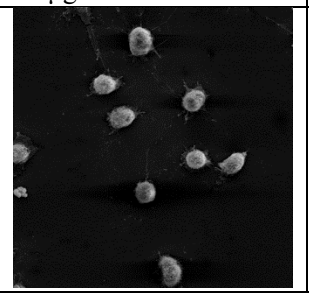
24 Hour Treatment with Drug					
Control 1064x	1 µg/mL 1064x	2 µg/mL 1064x	2.5 µg/mL 1064x	5 µg/mL 1064x	10 µg/mL 1064x
					

Table 2: Cells were treated with cytochalasin D in DMSO for 24 hours at concentrations of 1ug/mL, 2ug/mL, 2.5ug/mL, 5ug/mL, and 10ug/mL. Control cells were injected with only DMSO and viewed under SEM.

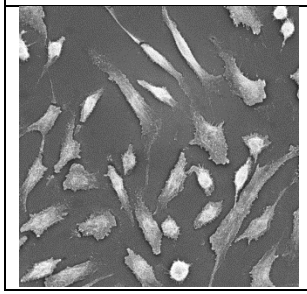
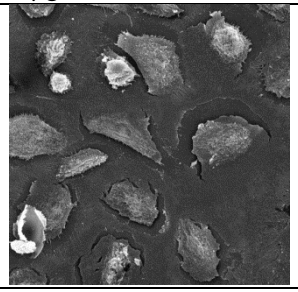
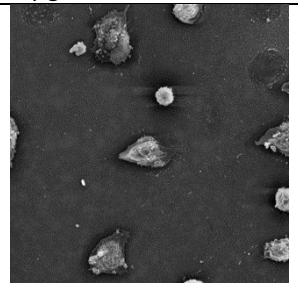
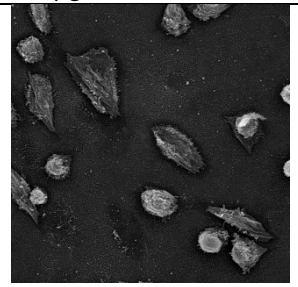
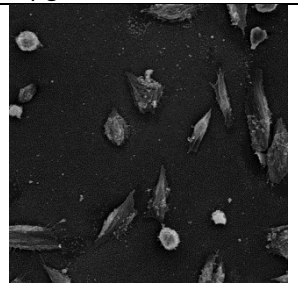
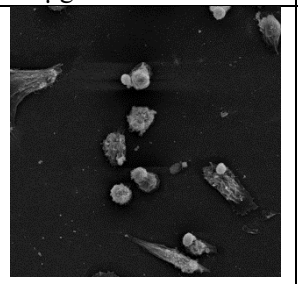
24 Hour Recovery from Drug					
Control 1064x	1 µg/mL 1064x	2 µg/mL 1064x	2.5 µg/mL 1064x	5 µg/mL 1064x	10 µg/mL 1064x
					

Table 3: After treatment with CD, cells were washed and fresh medium was added. Cells were allowed to recover for 24 hours and viewed using SEM

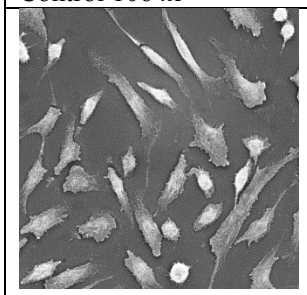
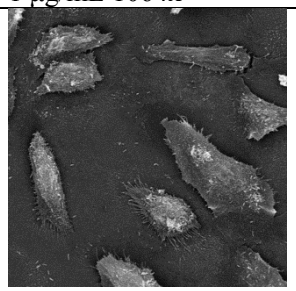
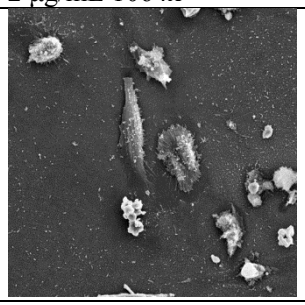
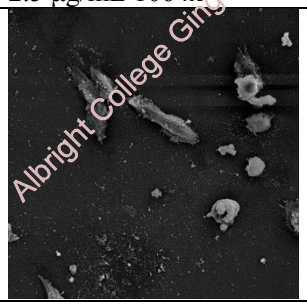
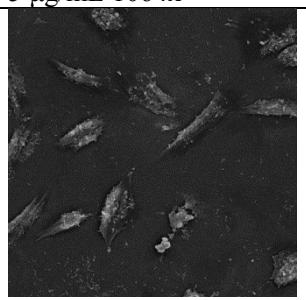
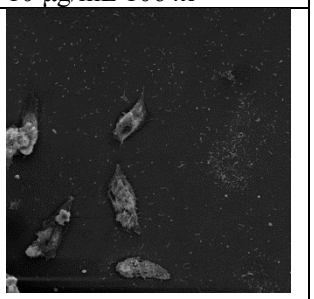
48 Hour Recovery from Drug					
Control 1064x	1 µg/mL 1064x	2 µg/mL 1064x	2.5 µg/mL 1064x	5 µg/mL 1064x	10 µg/mL 1064x
					

Table 4: After treatment with CD, cells were washed and fresh medium was added. Cells were allowed to recover for 48 hours and viewed using SEM

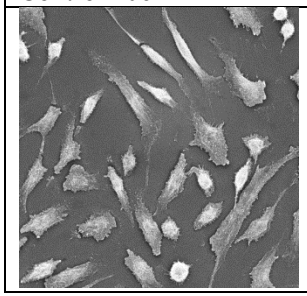
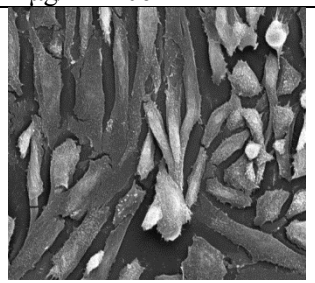
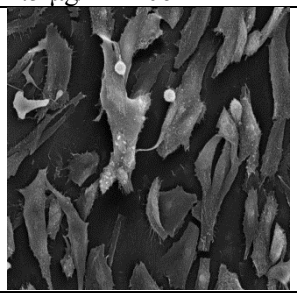
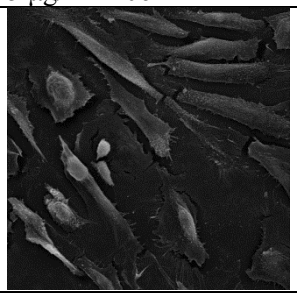
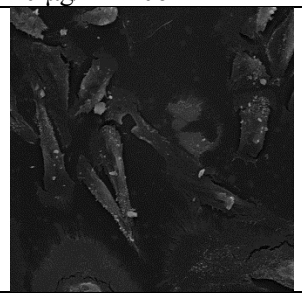
72 Hour Recovery from Drug					
Control 1064x	1 µg/mL	2 µg/mL 1064x	2.5 µg/mL 1064x	5 µg/mL 1064x	10 µg/mL 1064x
	Contamination was found				

Table 5: After treatment with CD, cells were washed and fresh medium was added. Cells were allowed to recover for 72 hours and viewed using SEM

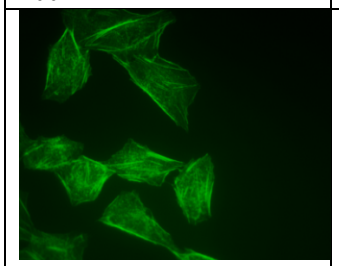
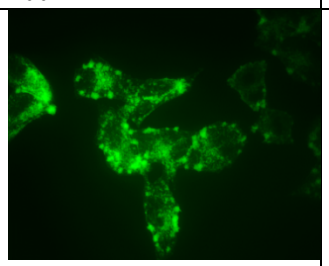
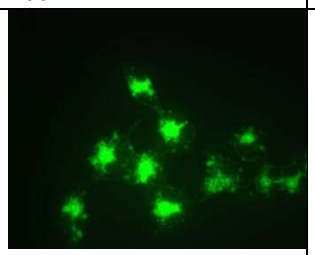
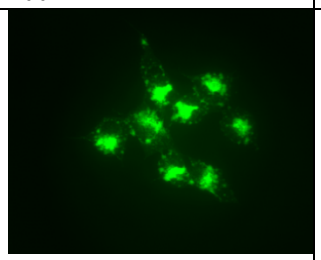
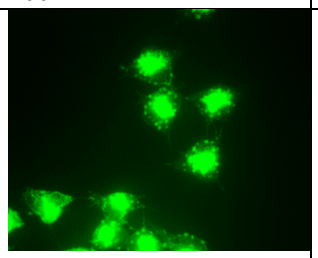
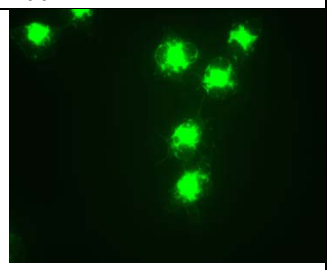
24 Hour Treatment with Drug					
Control	1 µg/mL	2 µg/mL	2.5 µg/mL	5 µg/mL	10 µg/mL
400x	400x	400x	400x	400x	400x
					

Table 6: Cells were treated with cytochalasin D in DMSO for 24 hours at concentrations of 1µg/mL, 2µg/mL, 2.5µg/mL, 5µg/mL, and 10µg/mL. Control cells were injected with only DMSO. Cells were prepared for fluorescence micrscopy by adding 185 uL of BODIPY FL phallacidin.

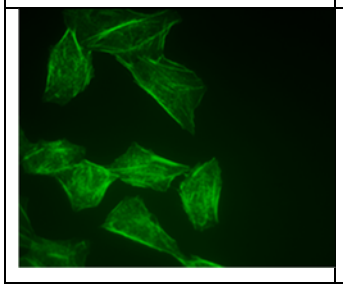
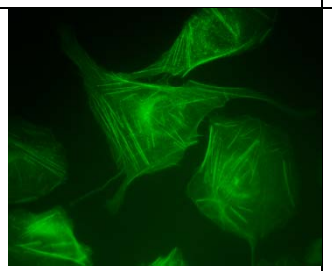
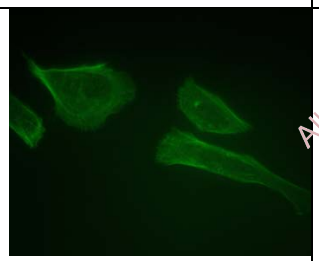
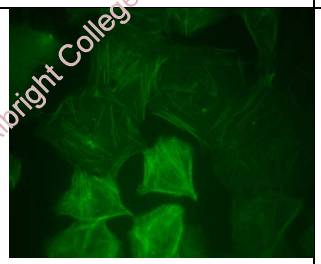
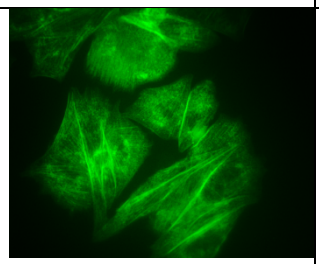
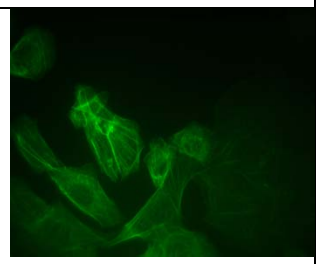
48 Hour Recovery from Drug					
Control	1 µg/mL	2 µg/mL	2.5 µg/mL	5 µg/mL	10 µg/mL
400x	400x	400x	400x	400x	400x
					

Table 7: After treatment with CD, cells were washed and fresh medium was added. Cells were allowed to recover for 48 hours and prepared for fluorescent microcopy by adding 185 uL of BODIPY FL phallacidin.

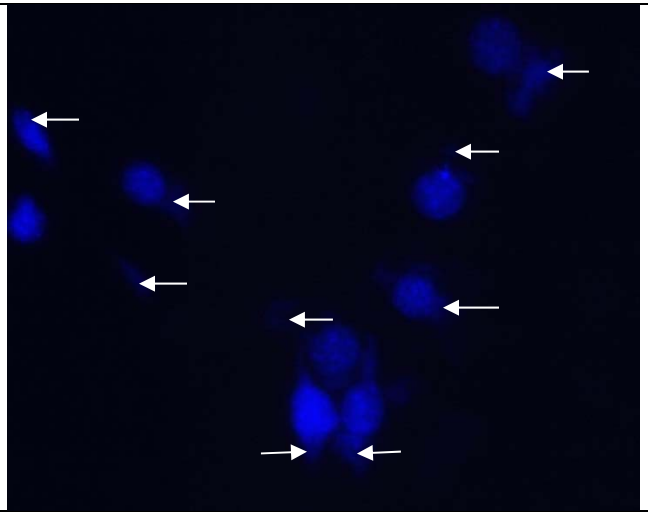
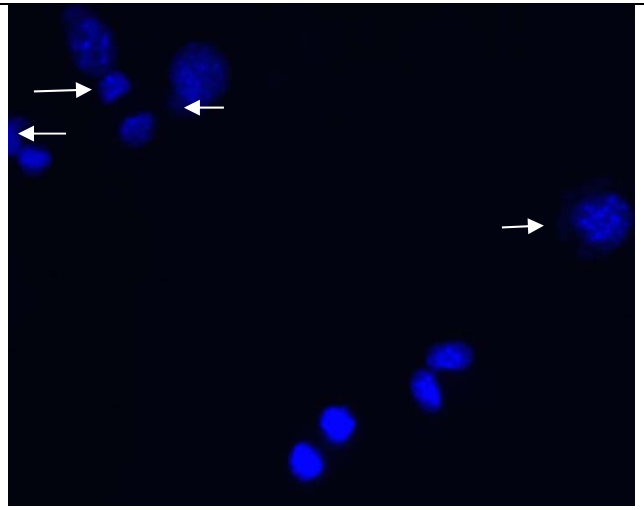
Virus Factory Production Comparison between cells infected with 24 hours of ECTV and cells with 24 hour ECTV and Cytochalasin D	
24 Hour ECTV	24 Hour ECTV and 24 hour 5 µg/mL of Cytochalasin D
400x	400x
	

Table 8: The presence of virus factories are indicated by a white arrow. Cells treated with virus and CD had a dramatic decrease in virus factory production.

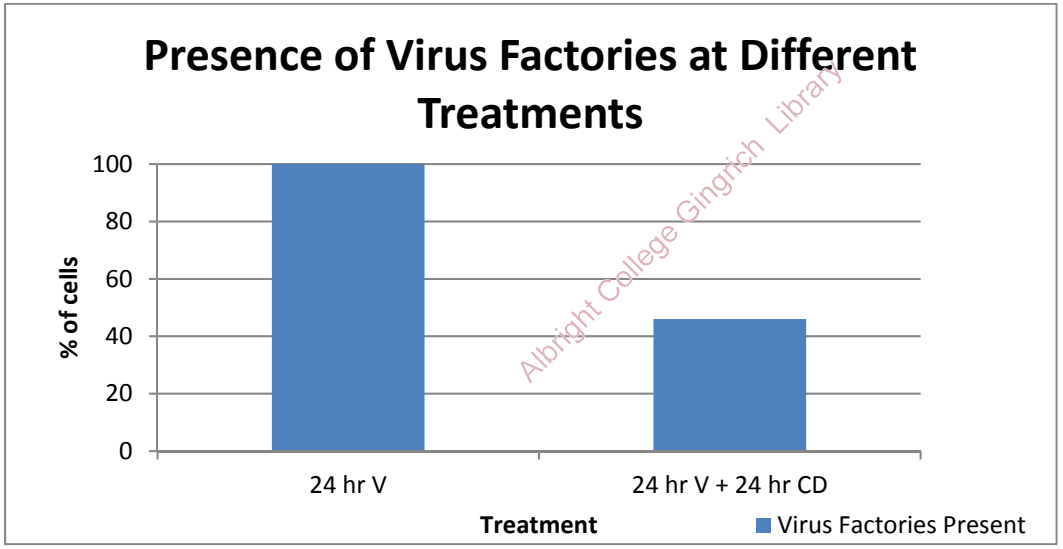


Figure 4: 50 random cells were counted for the presence of virus factories. 100% of cells treated with virus contained virus factories whereas only 23 cells out of 50 cells (46%) treated with ECTV and CD contained virus factories.

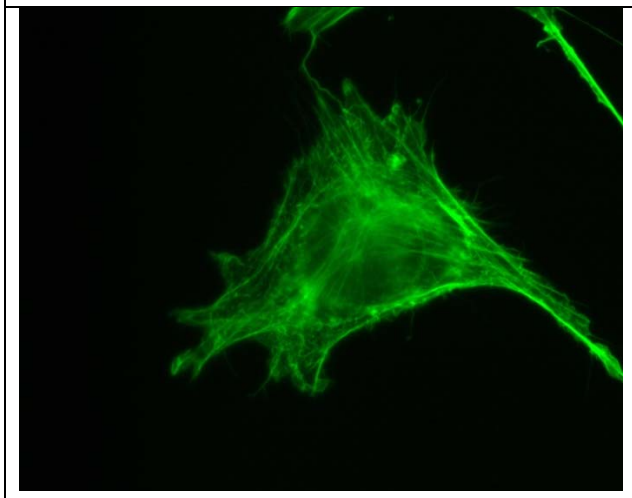
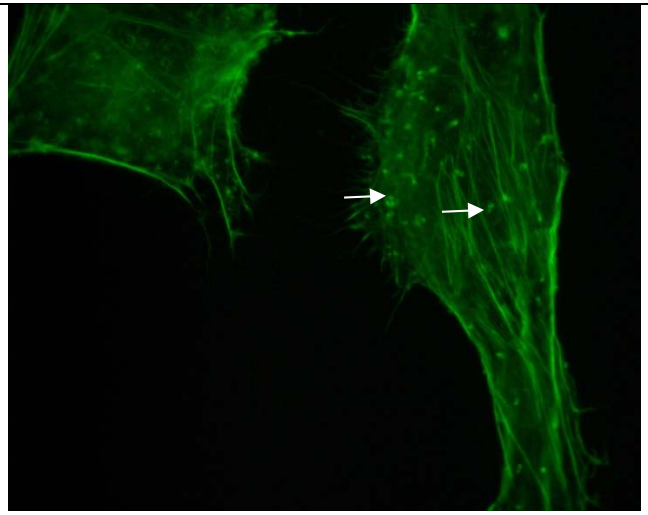
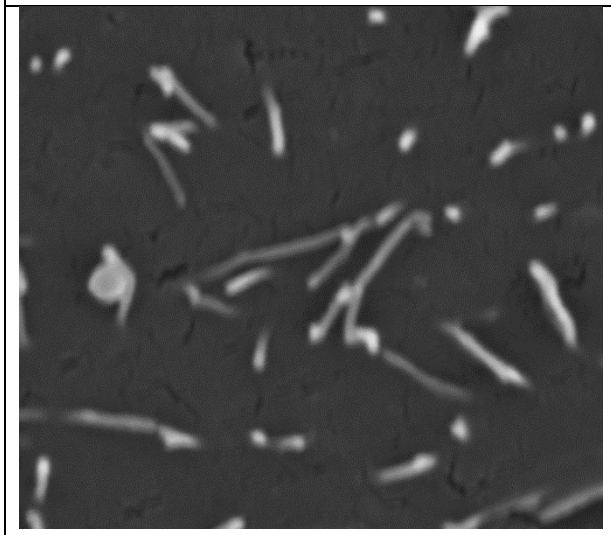
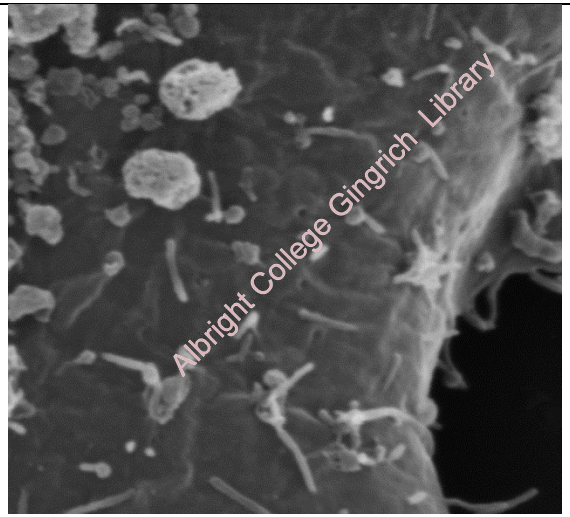
Actin tail formation upon treatment with 24 hour ECTV	
Control 1000x	24 hour ECTV 1000x
	
Control 19900x	24 hr ECTV 19900x
	

Table 9: Actin tail formation was seeing using both SEM and fluorescence microscopy for treatment with 24 hr virus

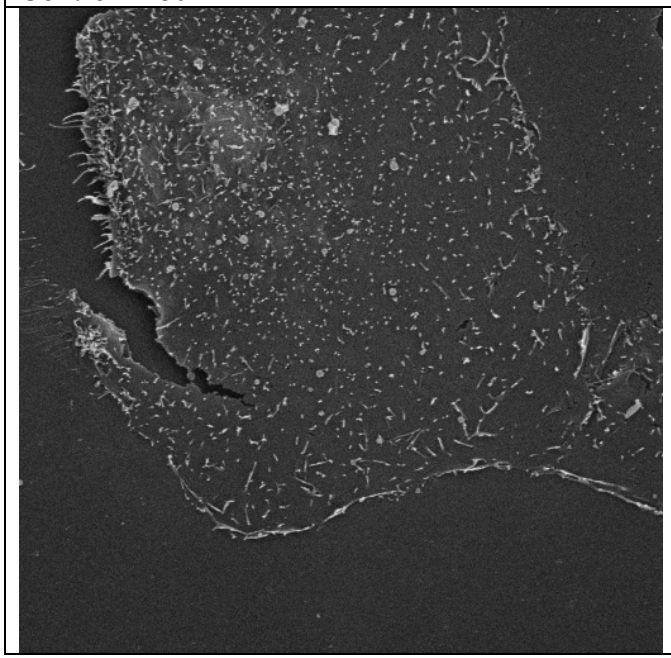
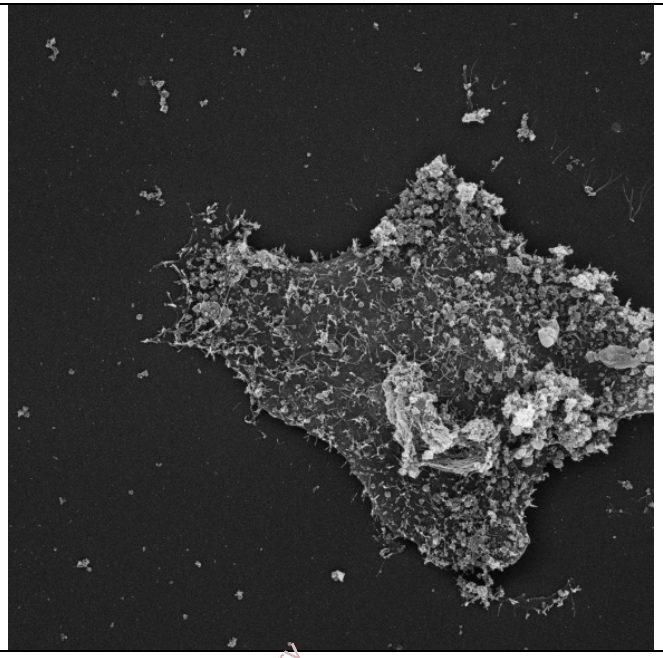
Process of budding on BSC-1 cells infected with ECTV	
Control 2480x	24 hr V 2480x
	

Table 10: The formation of blebs can be seen on the surface of cells treated with 24hr ECTV through the process of budding

CALCULATIONS:

Calculation 1:

number of viable cells: 17 + 17 + 26 + 15 + 25 + 17 + 17+ 26 + 15+ 25 = 200

number of non-viable cells: 0 + 3 + 1 + 2 + 2 + 0 + 3 + 1 + 2 + 2 = 16

Percent non-viable:

$$\frac{16}{200} = \frac{x}{100}$$

$x = 7\%$ nonviable, 93% viable

number of cells x 1000 (conversion of 1 mm^3 to 1 cm^3) x dilution factor x mL used = number of cells in total volume

184 x 1000 x 4 x 3= 22.08 x 10^5 cells in a total of 3mL

Want to seed 1 T-25 Flask with 1 x 10^5 cell/mL using 12 mL medium

12 mL x 1 flask = 12 mL --- 12(1 x 10^5) = 12 x 10^5 cells

Have: 22.08 x 10^5 cells/3 mL

Need: 12 x 10^5 cells/*x* mL

$$\frac{22.08 \times 10^5}{3\ \text{mL}} = \frac{12 \times 10^5}{x}$$

$x = 1.63$ mL cells, 10.37 mL medium

used 1.6 mL cells, 10.4 mL medium

Calculation 2:

number of viable cells: 33 + 42 + 58 + 55 + 33 + 33+ 42+ 58 + 55+ 33 = 442

number of non-viable cells: 1 + 2 + 2 + 0 + 1 + 1 + 2 + 2 + 0 + 1 = 12

Percent non-viable:

$$\frac{12}{442} = \frac{x}{100}$$

$x = 3\%$ nonviable, 97% viable

number of cells x 1000 (conversion of 1 mm^3 to 1 cm^3) x dilution factor x mL used = number of cells in total volume

430 x 1000 x 4 x 3= 51.60 x 10^5 cells in a total of 3mL

Want to seed 1 T-25 Flask with 1 x 10^5 cell/mL using 12 mL medium

12 mL x 1 flask = 12 mL --- 12(1 x 10^5) = 12 x 10^5 cells

Have: 51.60 x 10^5 cells/3 mL

Need: 12 x 10^5 cells/*x* mL

$$\frac{51.60 \times 10^5}{3\ \text{mL}} = \frac{12 \times 10^5}{x}$$

$$x = 0.697\ \text{mL cells}, 11.30\ \text{mL medium}$$

used 0.7 mL cells, 11.3 mL medium

Calculation 3:

number of viable cells: 50 + 58 + 72 + 79 + 70 + 50 + 58+ 72 + 79 + 70 = 658

number of non-viable cells: 5 + 4 + 3 + 4 + 5 + 5 + 4 + 3 + 4 + 5 = 42

Percent non-viable:

$$\frac{42}{658} = \frac{x}{100}$$

$$x = 6\%\ \text{nonviable}, 94\%\ \text{viable}$$

number of cells x 1000 (conversion of 1 mm^3 to 1 cm^3) x dilution factor x mL used = number of cells in total volume

616 x 1000 x 2 x 3= 36.96 x 10^5 cells in a total of 3mL

Want to seed 1 T-25 Flask with 1 x 10^5 cell/mL using 12 mL medium

12 mL x 1 flask = 12 mL --- 12(1 x 10^5) = 12 x 10^5 cells

Have: 36.96 x 10^5 cells/3 mL

Need: 12 x 10^5 cells/*x* mL

$$\frac{36.96 \times 105}{3\ \text{mL}} = \frac{12 \times 10^5}{x}$$

$$x = 0.97\ \text{mL cells}, 11.02\ \text{mL medium}$$

used 1 mL cells, 11 mL medium

Calculation 4:

number of viable cells: 45 + 42 + 38 + 56 + 42 + 45 + 42+ 38 + 56 + 42 = 446

number of non-viable cells: 1 + 3 + 2 + 1 + 0 + 1 + 3 + 2 + 1 + 0 = 14

Percent non-viable:

$$\frac{14}{446} = \frac{x}{100}$$

$x = 3\%$ nonviable, 97% viable

number of cells x 1000 (conversion of 1 mm^3 to 1 cm^3) x dilution factor x mL used = number of cells in total volume

432 x 1000 x 2 x 3= 25.92 x 10^5 cells in a total of 3mL

Want to seed 1 T-25 Flask with 1 x 10^5 cell/mL using 12 mL medium

12 mL x 1 flask = 12 mL --- 12(1 x 10^5) = 12 x 10^5 cells

Have: 25.92 x 10^5 cells/3 mL

Need: 12 x 10^5 cells/x mL

$$\frac{25.92 \times 105}{3\ \text{mL}} = \frac{12 \times 10^5}{x}$$

$x = 1.388$ mL cells, 10.61 mL medium

used 1.4 mL cells, 10.6 mL medium

Calculations 5:

Number of cells: 100,000

MOI: 5

100,000 x 5= 500,000

$$\left(8 * 10^7 \frac{\text{pFu}}{\text{mL}}\right) x = 500{,}000$$

$x = 0.00625\ mL$

$x = 6.25\ \mu L$

$use\ 7\ \mu L$ to ensure that excessice virus was present

LITERATURE CITED

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K., Walter, P. 2010. *Essential Cell Biology Third Edition*. New York: Garland Science. 590-97. Print.

Boratynska, A., Martyniszyn, L., Szulc, L., Krzyzowska, M., Szczepanowska, J., Niemialtowski, M..G. Contribution of Rearranged Actin Structures to the Spread of Ectromelia Virus Infection In Vitro. *Acta Virol* 54(1): 41-8. Web.

Cooper GM. *The Cell: A Molecular Approach*. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. Structure and Organization of Actin Filaments. Web. http://www.ncbi.nlm.nih.gov/books/NBK9908

Goddette, D. W., Frieden, C. 1986. Actin Polymerization The Mechanism of Action of Cytochalasin D. *The Journal of Biological Chemistry* 261.34: 15974-15980. Web.

Godman, G. C., Miranda, A. F., Deitch, A. D., Tanenbaum, S. W. 1975. Action of Cytochalasin D on Cells of Established Lines III. Zeiosis and Movements at the Cell Surface. *The Journal of Cell Biology* 64: 644-667. Web.

Malfarelli, F., King, N.W., Yagi, M.J. 1987. Effects of Cytoskeletal Disrupting Agents on Mouse Mammary Tumor Virus Replication. *Virus Res* 7(4): 281-295. Web.

Miranda, A. F., Godman, G. C., Deitch, A. D., Tanenbaum, S. W. 1974a. Action of Cytochalasin D on Cells of Established Lines I. Early Events. *The Journal of Cell Biology* 61: 481-500. Web.

Miranda, A. F., Godman, G. C., Deitch, A. D., Tanenbaum, S. W. 1974b. Action of Cytochalasin D on Cells of Established Lines II. Cortex and Microfilaments. *The Journal of Cell Biology* 62: 406-423. Web.

Mockrin, S. C., Korn, E. D. 1983. Kinetics of Polymerization and ATP Hydrolysis by Covalently Cross-linked Actin Dimer. *The Journal of Biological Chemistry* 258.5: 3215-3221. Web.

Redtke, K., Dohner, K., Sodeik, B. 2006. Viral Interactions with the Cytoskeleton: a hitchhiker's guide to the cell. *Cellular Microbiology* 8: 387-400. Web.

Schliwa, M. 1982. Action of Cytochalasin D on Cytoskeletal Networks. *The Journal of Cell Biology* 92: 79-91. Web.

Schramm, B., Locker, J.K. 2005. Cytoplasmic Organization of POXvirus DNA Replication. *Traffic* 6: 839-846. Web.

Taylor, M.P., Koyuncu, O.O., Enquist, L.W. 2011. Subversion of the Actin Cytoskeleton During Viral Infection. *Nat Rev Microbiol* 9(6): 427-439. Web.