The Role of Myosin Phosphorylation on Bivalent Oscillations in *Mesostoma ehrenbergii* Primary Spermatocytes

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Abstract

The main aspect of my thesis looks at the role of myosin phosphorylation and total myosin inhibition on bivalent oscillations during prometaphase in primary spermatocytes of *Mesostoma ehrenbergii*. Previous studies have shown that myosin is required for proper chromosome movements in a variety of different cell types. I concentrated on increasing and decreasing the activity of myosin through inhibiting different phosphorylation pathways as well as directly inhibiting myosin. I studied the effects of enhancers and inhibitors of myosin activity on chromosome oscillations. The experiments showed that there are redundant pathways for phosphorylations of the myosin involved in spindle force production. My thesis also looks at the effects of myosin phosphorylation on distance segregation of univalents. Pharmacological studies enhancing or inhibiting myosin showed that myosin may be involved in the number of univalent movements during prometaphase, that a single phosphorylation pathway is involved, and that there are additional pathways that contribute to these movements.

Overall, my results in *Mesostoma ehrenbergii* primary spermatocytes suggest that myosin is involved in chromosome oscillations during prometaphase and distance segregation of the univalents. For all of these characteristics, there are redundant phosphorylation pathways and mechanisms that contribute to these complex processes.

To Nicole and Nicham

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Candidate's contributions:

The Role of Myosin Phosphorylation on Bivalent Oscillations in *Mesostoma ehrenbergii* Primary Spermatocytes

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- Majority of treatments of *Mesostoma ehrenbergii* primary spermatocytes with different myosin phosphorylation inhibitors and enhancers
- Analyzing prometaphase bivalent oscillations towards and away from the pole
- Writing and preparing the final manuscript

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General Introduction

The main purpose of my thesis is to study the forces that contribute to chromosome movement during cell division. Specifically I look at chromosome movement during prometaphase in meiosis in primary spermatocytes. The mechanisms within the cell that allow for these complex movements during meiosis have been extensively studied for many years. To this day however the exact mechanisms and proteins involved are still elusive and debated. Most models attribute the main force production to microtubules and its associated motor proteins. There are some inadequacies with this model which will be described in a later section. To help try and explain these inadequacies, I looked at myosin as a potential major force contributor for chromosome movements. To start off I will briefly describe the different stages of meiosis.

A. Conventional Meiosis

Cells can divide and reproduce in one of two ways; mitosis or meiosis. Mitosis generally occurs in somatic cells and produces two identical diploid daughter cells. Meiosis is the process that occurs in sex cells and produces 4 non-identical haploid sex cells. It is the basis of sexual reproduction in eukaryotic organisms. Both processes are similar but meiosis only has one round of chromosome replication followed by two successive cell divisions (Morgan, 2007). Meiosis I results from the division of homologous chromosomes while meiosis II results in the division of sister chromatids

(Morgan, 2007). Meiosis I and II are divided up into 5 distinct phases: prophase, prometaphase, metaphase, anaphase, and telophase.

A.1 Prophase I

Prophase I is typically the longest phase during meiosis and is when chromosomes condense and become visible under white light. During this stage, homologous recombination occurs between homologous chromosomes. Recombination along with crossover allows for genetic variation and the proper pairing of chromosomes (Meier & Gartner, 2006; Burgoyne & Mahadevaiah, 2007). The chromosomes at this stage are called bivalents which consist of two chromosomes, composed of two chromatids each. Once the chromosomes have been properly paired, non-sister chromatids can crossover creating chiasmata (Roeder & Baillis; 2000; Hochwagen & Amon, 2006).

A.2 Prometaphase I

Before transitioning into metaphase the cell enters a stage called Prometaphase. In this stage, the nuclear membrane breaks down into many membrane vesicles (Pickett-Heaps et al., 1984). The newly formed vesicles begin to surround the chromosomes and large protein structures called kinetochores form on the chromosomes. Once the nuclear membrane breaks down, signs of the spindle forming can be observed. In animal cells, microtubules which are prominent in the spindle, begin to extend from the centrosomes (Pickett-Heaps et al., 1984). Once the kinetochores are captured by the microtubules the chromosomes begin to move (Pickett-Heaps et al., 1984). The chromosomes can begin to move in a agitated fashion to and from poles,

but these movements are irregular. These irregular and random movements will eventually bring the chromosomes to the center of the cell where they line up on the metaphase plate (Pickett-Heaps et al., 1984).

A.3 Metaphase I

Metaphase is the stage where “random” chromosome movement stops and the paired homologous chromosomes align along the equatorial plane of the cell between the poles called the metaphase plate (Page & Orr-Weaver, 1997; Tyson & Novak, 2008). Spindle fibers extend from the centrioles at the poles and attach to the chromosomes. Kinetochores on sister chromatid pairs attach to the same pole allowing for them to be segregated together. The kinetochores on homologous chromosomes face opposite poles allowing them to be separated to different ends of the cell during anaphase. The bivalents continue to experience tension from the kinetochore fibers but will not detach until all bivalents have aligned (Morgan, 2007). Proper alignment and attachment of the chromosomes in this step is crucial. If there is any misalignment of any of the chromosomes, checkpoint delays will occur until the problem is rectified (Cleveland et al., 2003). Even though microtubules are dynamic, the spindle maintains a specific length until the start of anaphase.

A.4 Anaphase I

Once all of the homologous chromosomes have properly, chromosome segregation is ready to begin. The loss of cohesion between homologous chromosomes allows the chromosomes to begin segregation. Anaphase consists of two different stages: Anaphase A and Anaphase B. In Anaphase A, kinetochore

microtubules shorten and begin the initial poleward movement of chromosomes to the poles (Karp, 1999). Anaphase B consists of the actual separation of the spindle poles and occurs once the homologous chromosomes have separated some small distance. In fact, some cells have only elongation, some only have movement to the poles, and some a mixture. There are many models that describe how the chromosomes are separated and moved to opposite poles as will be discussed later. The separation of the spindle poles is thought to be a result of the sliding of interpolar microtubules in opposite directions (Sharp et al., 1999). A motor protein called kinesin-5 cross-links the plus ends of the interpolar microtubules and pushes them poleward. The contribution of Anaphase A and B in chromosome segregation varies depending on the cell type. For example, in yeast cells, Anaphase B is primarily used to separate chromosomes causing spindles to increase in length by almost 15 times (Hagan & Hyams, 1996).

A.5 Telophase I

Once chromosomes have properly segregated to their respective poles, telophase can begin. The first step of telophase is for the mitotic spindle to be broken down. This is then followed by fragments of the nuclear envelope to starting to associate with the chromosomes (Morgan, 2007). These nuclear fragments then begin to fuse and reform the nuclear membrane.

A.6 Cytokinesis

In the final phase of Meiosis I, the cytoplasm is separated in cytokinesis. During cytokinesis in animal cells, a cleavage furrow forms. It quickly spreads around the entire cell and deepens until the two cells have been divided (Morgan, 2007). The mechanism

that allows this process to occur is a contractile ring. The contractile ring is composed of both actin and myosin along with many structural proteins. This contractile ring forms where the metaphase plate used to be (Morgan, 2007). As the ring contracts new membrane is placed by the ring allowing for two new daughter cells to form. Meiosis II then begins and proceeds through the same steps as Meiosis I but there is no S phase. In Meiosis II, sister chromatids are separated instead of homologous chromosomes forming 4 haploid daughter cells.

B. Meiosis in Other Organisms

The meiosis described above is the textbook version taught to students and occurs in humans and many model organisms such as yeast. Meiosis, however, can occur in many different ways while still achieving the same result. The primary spermatocytes that I study do not fit in with the conventional form of cell division for germ cells, so I will provide examples of different organisms that also do not follow the conventional pattern of meiosis. In *Sciara coprophila*, a fungus gnat, male meiosis is strikingly different from the conventional meiosis (Kubai, 1982). As previously stated, chromosome pairs line up along the metaphase plate and are attached to opposite poles through spindle fibers creating a bipolar spindle. In the fungus gnat, there is, however, a monopolar spindle that has been observed in many insects (Schrader, 1953).

In prophase of the male fungus gnat, maternal and paternal chromosomes are spatially separated (Kubai, 1982). As the cell progresses into metaphase, only one polar complex forms at a single pole. Kinetochore microtubules radiate towards the paternal

set of chromosomes. Instead of pulling the chromosomes to the pole they seem to push the paternal chromosomes further from the pole. The maternal set has no visible microtubule attachment but still segregates to the single pole (Kubai, 1982). A third set of chromosomes called limited chromosomes can be seen closely associated with the maternal chromosomes. These chromosomes also do not have any visible kinetochore microtubules and only stay near the pole but do not segregate towards it.

Another example of an organism which has a very different way of completing meiosis is *Poeciliopsis*, a viviparous all-female fish. Egg production in this fish is similar to that of the fungus gnat in that the spindle is monopolar (Cimino, 1972). Maternal chromosomes in this case, however, have kinetochore microtubules which attach to the single pole. Since the homologs only consist of maternal chromosomes, synapsis does not occur. During anaphase, the maternal set of chromosomes is pulled towards the pole. The paternal set, in this case, is not attached to any spindle fibers. Instead, they are dispersed randomly throughout the cytoplasm and eventually aggregate at the opposite end of the cell (Cimino, 1972). Cytokinesis at this point is inhibited resulting in an oogonium with two separate clusters of chromosomes. Meiosis II continues and a haploid polar body is expelled from the cell. The paternal set either disintegrates or is reabsorbed into the ooplasm or the paternal set is pinched off in a bud (Cimino, 1972).

C. Distance Segregation

One of the biggest challenges in cell division is to ensure that paired chromosomes are segregated correctly, allowing for each new daughter cell to receive a copy. In the conventional meiosis as described above this problem is dealt with by

linking the chromosome pairs together through chiasmata or other forms of chromosome adhesion. In many organisms however the proper sorting of chromosomes is done without obvious connections between the chromosome pairs. Distance segregation is a highly variable process and the segregation of these partners could be at different times compared to other pairs in the cell (Brady & Paliulis, 2015). In some cells the partners separate once the nuclear membrane has broken down (Hughes-Schrader 1969, 1983; Virkki, 1971), while in other cells these partners segregate once all other bivalents have separated to different poles (Forer, 1980; LeCalvez, 1947).

These chromosomes are not only variable in when they segregate but they also vary on how they attach to the spindle. The sister kinetochores can either attach to the same pole or they can attach to opposite poles (Brady & Paliulis, 2015). These two types of attachment are not exclusive as a single cell can have both types of attachment present. In many organisms distance segregation tends to occur in sex chromosomes. There are, however, examples of distance segregation occurring in autosomes. Some of these organisms include liverwort and bugs belonging to Hemiptera and Coreidae (Lorbeer, 1934).

D. *Mesostoma ehrenbergii ehrenbergii*

For my thesis, I studied primary spermatocytes obtained from *Mesostoma ehrenbergii*. The male meiosis that occurs in this organism is very different from the conventional meiosis, but this is not uncommon as shown by the examples above. I use this organism in order to explore the role of myosin phosphorylation and activity during

prometaphase chromosome movements. I will now describe the male meiosis in primary spermatocytes and elucidate the advantages of studying this organism.

Mesostoma ehrenbergii is a transparent hermaphroditic flatworm belonging to the class Turbellaria and order Rhabdocoela (Fuge, 1987). The aquatic flatworm is able to produce offspring in one of two different ways. They can either produce viviparous eggs or diapausing eggs. Like other flatworms in the Typhoplanidae family, this is a common occurrence. The diapausing eggs have thick shells and are rich with yolk since they have a dormant phase prior to hatching (Ferguson & Hayes, 1941). The viviparous eggs, on the other hand, are thin shelled with little yolk and they develop quickly in uteri. The flatworms can either produce viviparous eggs before diapausing eggs or only produce diapausing eggs.

D.1 Meiosis in *Mesostoma ehrenbergii*

In the male primary spermatocytes meiosis, there are 3 large bivalents and 4 univalents (2n=10) (Oakley & Jones, 1982). Flattened cells are approximately 30 um in length with an approximate bivalent length of 20-25 um (Ferraro-Gideon, Hoang & Forer, 2014). The bivalents only have one chiasmata each and are all metacentric so there are visible arms. The four univalents form pairs where two are metacentric and the other two are acrocentric. These morphological traits are not consistent for all *Mesostoma ehrenbergii* species and depend on the region they are from (Ferraro-Gideon, Hoang & Forer, 2014; Husted et al. 1939; Husted and Ruebush, 1940; Hebert and Beaton 1990). The population that I study in the lab are from Lake Rondeau in Ontario, Canada. Populations from Germany have larger primary spermatocytes

approximately 40 um in length and bivalent lengths of 27-40 um (Oakley 1985; Fuge 1987).

D.1.1 Rapid Kinetochore Movements

During early Prometaphase I, some of the bivalents may have both kinetochores oriented towards one pole (Fuge, 1989). As the cell progresses through Prometaphase I, a single kinetochore will reorient to face the opposite pole. Once the bivalent has achieved this orientation it oscillates back and forth between the poles. The oscillations can occur for about 1-2 hours before the onset of anaphase (Ferraro-Gideon et al., 2013). Two distinct movements have been observed when looking at the bivalent oscillations. The movement of the bivalents can either be in-phase or out of phase. When the movement is in phase, both sister kinetochores will either move towards or away from the pole at the same time (Fuge, 1989). This causes the bivalent to become stretched when both kinetochore move towards the pole or shortened when both kinetochore move away from the pole. The bivalent is not displaced during in-phase movement. When the movement is out of phase one kinetochore will move towards the pole while the other moves away from the pole. This motion causes actual displacement of the bivalent and maintains length. The bivalents also may not oscillate for the entire time. Sometimes one kinetochore will pause for a short amount of time before resuming movement (Fuge, 1989). Other than the initial kinetochore reorientation observed during early prometaphase I, bivalents have been observed to reorient.

D.1.2 Distance Segregation in *M. ehrenbergii*

During Prophase and Prometaphase, four univalents can be observed. These univalents are in pairs, two metacentric and two acrocentric. When the spindle is being formed the univalents move to the poles. The univalents tend to stay at the poles for the majority of Prometaphase but can move between poles (Oakley, 1985). The placement of the univalents can vary depending on the stage at which the cell is in. There are 5 different possible configurations that the univalents can be found in, e.g. two univalents at each pole, one univalent at a single pole and three at the other, etc. Oakley calculated the probability of each distribution if the univalents were randomly at ay pole and found actual values to be very different. The number of cells with one metacentric and one acrocentric univalent at each pole was found in 2x more cells than expected suggesting that this configuration is actively brought about (Oakley, 1985). As the cell actively works to maintain this specific distribution, Oakley thought that the movement of the univalents is not random. When looking at live cell preparations homologous pairs of either metacentric or acrocentric chromosomes have been observed to switch places with each other, further supporting that non-random segregation occurs (Oakley, 1985). Examples of non-random segregation are abundant including mealy-bugs (Nur, 1982; Schrader 1921) and *Sciara* (Gerbi, 1986).

D.1.3 Precocious Cleavage Furrow

In cells that follow the conventional meiosis, the cleavage furrow only appears during cytokinesis when the cell is ready to divide. In *Mesostoma* primary spermatocytes the cleavage furrow begins to form in prometaphase once all of the

bivalents have become bipolarly oriented. After the furrow begins to ingress it eventually pauses. As the cell progresses and gets closer to anaphase the furrow tightens a bit more. Furrow ingression only completes once anaphase has completed and all chromosomes have segregated properly (Forer & Pickett-Heaps, 2010). This is not common since in conventional cells signals that allow the cleavage furrow to form only occur once the cell has completed anaphase. Another important feature of the furrow is that it is able to move along the length of the cell. When univalents move from one pole to another the furrow will move closer to the pole that has fewer chromosomes (Forer & Pickett-Heaps, 2010). The furrow does not only move if a univalent excursion occurs but also moves when bivalent reorients.

D.1.4 Absence of Metaphase

In conventional cell types, the cell normally proceeds into metaphase after prometaphase and prior to anaphase. In *Mesostoma ehrenbergii* primary spermatocytes, this is not the case. There has been disagreement about whether the stage where the bivalents are bipolarly oriented in *Mesostoma* primary spermatocytes is prometaphase or metaphase (Fuge, 1989). Metaphase is generally described as the stage where the chromosomes have lined up on the metaphase plate. The 3 large bivalents could be said to adopt this position, but the univalents never do. Oakley (1985) suggested that instead, the univalents are in the correct distribution before anaphase, but that the present phase is still referred to as metaphase. The movements that the bivalents adopt correspond to prometaphase movements observed in other species (Dietz, 1956; 1969). Although the bivalents appear to be aligned on the metaphase plate that may just be due to the size of the bivalents relative to the size of the cell.

E. Components of the Spindle

The spindle apparatus is a complex cytoskeletal structure that is formed both during mitosis and meiosis in order to properly segregate genetic material. When the spindle is formed during meiosis it is referred to as the meiotic spindle and is composed of hundreds of different proteins. Prior to 1952 the existence of the spindle in mitotic and meiotic cells was debated. Up to this point the fibres were unable to be viewed in live cells and were considered to be an artifact that occurred when cells were fixed. In 1952, Inoué used birefringence in order to visualize the spindle in live cells of the marine annelid, *Chaetopterus pergamentaceous*. This has led to further studies in order to determine the composition and role of the various proteins involved. For my thesis I am studying the possible roles of myosin within the spindle and how it contributes to different movements. Current models however favour microtubules as being the major force producers, so I will explain them in the next section.

E.1 Microtubules

Microtubules can be found throughout the cytoplasm and are a major cytoskeletal component. They are highly dynamic structures that can grow up to 50 micrometers in length. Microtubules are involved in many cellular processes and cell movements such as flagella beating and intracellular transport (Bray, 2001). As a polymer, they form a hollow tube with a diameter approximately 24 nm (Karp, 1999). The hollow tubes are formed from the polymerization of alpha and beta tubulin. Due to the arrangement of the alpha and beta tubulin dimers, the microtubule has polarity. The end of the microtubule where the beta monomer is exposed is considered the plus end

and the site where the polymer actively polymerizes. The opposite end is the minus end and is the end where the polymer actively depolymerizes (Wade, 2009; Jiang & Akhmanova, 2011).

Experiments have determined that the microtubules are nucleated by the microtubule organizing center (MTOC) with the plus end extending to the kinetochores (Wittmann et al., 2001; Mcintosh et al., 2002). The polymerization of the tubulin dimers is mediated by the binding of guanosine triphosphate (GTP). When the dimer is incorporated into the structure GTP remains bound to the alpha-tubulin monomer, while the GTP bound to the beta-tubulin monomer is hydrolyzed. The release of energy causes the stability of the microtubule to decrease and leads to the change in growing or shortening at the ends (Bray, 2011).

The ability of the microtubules to be able to lengthen or shorten is an important factor in the meiotic spindle. The dynamic ability and stability of microtubules are governed by a number of different proteins such as microtubule associated proteins (MAPs) and katanin (Heald &Nogales, 2002). MAPs contribute to the overall stability of the microtubule and promote growth. Katanin, on the other hand, is a severing enzyme that contributes to microtubule instability by creating new ends prone to depolymerization. The dynamic properties allow the microtubules to contribute to spindle formation and other processes during meiosis.

E.1.1 Motor Proteins

Dynein is one of the motor proteins that assist microtubules in completing many of the processes during meiosis. Dynein is a motor protein that walks along

microtubules and utilizes adenosine triphosphate (ATP) as an energy source. Dynein moves towards the minus-end on the microtubule which is in contrast to the motor protein kinesin. There are two different classes of dyneins called ciliary and flagellar dyneins (Lodish et al., 2000). Of the two different classes, cytoplasmic has been found to have a greater importance during meiosis and has been localized in the spindle of *Caenorhabditis elegans* (Pfarr et al., 1990). This class of dyneins always has at least one stalk attached to a microtubule allowing it to travel longer distances before detaching.

Kinesin is another motor protein found to be strongly associated with microtubules in the spindle. Kinesins are a class of ATPases that walk along microtubules in a plus end directed manner. The motor protein consists of two motor subunits and two light chains (Vale, Reese & Sheetz, 1985). Kinesins have been found to play an important role in maintaining proper spindle length as well as being required to slide microtubules during Prometaphase and depolymerization during anaphase (Goshima & Vale, 2005).

E.1.2 Microtubule Mechanisms Contributing to Anaphase Movement

During chromosome segregation paired homologous chromosomes or sister chromatids are separated and migrate to opposite ends of the pole. It is essential that this process is completed properly in order to facilitate cell replication. Exactly how chromosomes are moved to the poles during either mitosis or meiosis is not well understood. Many theories of movement rely solely on microtubules as being the main

component and major force producer, the major theories include the Flux model and Pac-Man model.

E.1.2.1 Flux Model

Microtubules are able to polymerize at the plus end where the kinetochores attach and depolymerize at the minus end where the MTOC resides. If the rates of these two processes are the same, it is known as microtubule treadmilling. Injection of fluorescently tagged tubulin shows the subunits add to the plus end and travel along the microtubules before depolymerizing at the pole (Mitchinson et al., 1986). When the rates of these two processes are the not equal however this causes the microtubule to shorten. When the decrease in length is as a result of depolymerization at the pole, this is known as microtubule flux (Rogers, Rogers, & Sharp, 2005). This mechanism is common in many cell types and has been found in all metazoan cells.

In order for flux to take place in the cell properly, 3 activities must occur. Eg5 is a plus-end directed kinesin that cross links and slides microtubules during flux. Experiments using *Xenopus* eggs by Miyamoto and colleagues (2004) showed that when this protein was inhibited the flux rate decreased. Secondly, microtubules must depolymerize at the minus-end by a family of kinesin-13 proteins. Experiments have shown that inhibition of KLP10A or Kif2a, the Drosophila, and human homologs, significantly reduces microtubule flux (Ganem & Compton, 2004; Rogers et al., 2004). Lastly, microtubule plus-ends bound to the kinetochores must still polymerize and it was found in Drosophila S2 cells a CLIP-associated protein was found to be a critical component (Maiato, Khodjakov & Rieder, 2005).

Although flux has been thought to play a major role in chromosome segregation, this may not be the case for all cell types. In a study by Rogers and colleagues (2003), microtubule flux was inhibited by adding inhibitory antibodies for KLP10A which is required for flux after which the speed of the chromosomes reduced by 2.2 um/min which is consistent with the speed of microtubule flux towards the poles. Since the chromosomes were still able to segregate to poles, it is an indication that microtubule flux is not the only mechanism responsible for these movements.

E.1.2.2 Pac-Man Model

The previous model suggested that the kinetochores are passive and kinetochore microtubules are responsible for chromosome movement. In the Pac-Man model, the kinetochore is actively involved in chromosome movement and is highly reliant on microtubule dynamics. The Pac-Man model differs from the traction fiber model in that the kinetochore is no longer a passive entity. The role of the kinetochore is instead to chew up the plus-end of the microtubule while remaining attached causing the chromosomes to segregate to the poles (Cassimeris et al., 1988; Koshland et al., 1988; Nicklas, 1989).

In order to determine whether microtubules depolymerize at the kinetochore, cells were injected with x-rhodamine or fluorescently labeled tubulin (Gorbsky et al., 1987; 1988). Once the labeled tubulin was incorporated into the kinetochore, an area of microtubules was photobleached once anaphase started. As the chromosomes moved towards the pole, the photobleached area in the microtubules did not move in relation to the pole (Gorbsky et al., 1987; 1988). Since the photobleached area did not move

closer to the pole this indicates that the microtubules were depolymerizing at the kinetochore.

This process can only occur with the assistance of other proteins since the kinetochore itself does not cause the actual depolymerization of the microtubule. In a study by Rogers and colleagues (2004), a microtubule destabilizing KinI kinesin enzyme was targeted for inhibition. They found in *Drosophila*, that when KLP59C was inhibited, depolymerization of the microtubules at the plus-end was greatly diminished.

Although this model has been widely accepted, few acknowledge significant phenomena that refute this model. For example, in diatoms, very few microtubules actually insert at the kinetochore. Instead the kinetochores actually attach to a collar (Tippit and Pickett-Heaps 1977; Pickett-Heaps et al., 1978). Since the Pac-Man model and other microtubule models state that the microtubules terminate at the kinetochore this piece of evidence weakens the model. In addition, at the end of anaphase chromosomes have been observed to move past the end of the spindle, which cannot be explained through microtubule based models.

In an experiment by Zhang and Nicklas (1996), chromosomes and their kinetochores were surgically removed from the cell. Even in the absence of the chromosomes the kinetochore fibres shortened at a speed consistent with normal anaphase. This suggests that the shortening of the fibres does not require the presence of the kinetochore and is accomplished by other proteins within the spindle causing chromosomes to move.

The inadequacies and anomalies described in the microtubule based models above show that there are other components within the spindle that contribute to chromosome movements during cell division. It has been suggested that other models such at the spindle matrix and actin and myosin based models collaborate with microtubules in the spindle to allow for cell division to occur. I will now cover these models as evidence to support this conclusion.

E.2 Spindle Matrix Model

For many years the existence and composition of the spindle has been studied extensively. Despite all of the research and time that has gone into studying the spindle, much of it still remains elusive (Pickett-Heaps et al., 1982; 1997; Scholey et al., 2001). Many agree that microtubules are the main component and force producers but others have shown that there is a large array of proteins that work together in order to form the spindle and contribute to force production. Despite the large number of experiments that have shown many proteins involved in spindle formation regulation and chromosome movement, the topic is still quite debatable among scientific colleagues.

In an evolutionary perspective, it is suggested that as the spindle evolved it would contain components of the interphase cell as well as other primitive motility systems (Pickett-Heaps & Forer, 2009). Other systems then came together in order to form the redundant reliable mitosis seen in current spindles. These redundancies in the spindle make it very difficult to characterize and explain how the spindle functions. As a result straightforward interpretations have turned out to be consistently wrong or inadequate. For example in the 60s and 70s it was assumed that the kinetochore was a

microtubule organizing centre allowing for the formation of kinetochore fibres. This conclusion was later proved incorrect as the kinetochore microtubules can also originate from the pole (Wadsworth and Khodjavok 2004; Rieder 2005). The issue continues to become increasingly complex as experimenters disregard all other potential candidates that can attribute to the formation of the spindle as well as produce the force required for complex chromosome movements.

It has been proposed that non-microtubule components of the spindle may be involved in generating force during anaphase A (Pickett-Heaps et al., 1996; Spurck et al., 1997). Observations in live cells have shown that microtubules attach to the kinetochore fibres and extend to the poles, so the assumption has been made that they produce force for movement. From a number of experiments it has been suggested that although microtubules are central to chromosome movements, other proteins can be working with them. In experiments by Pickett-Heaps et al (1996) and Spurck et al. (1997), kinetochore microtubules were cut during anaphase and chromosomes were still able to move, with some accelerating. What can be interpreted from this is that kinetochore microtubules may actually inhibit the poleward movement of chromosomes and act as a regulating force. It is likely that an additional system, such as the spindle matrix produces force to move chromosomes and that the force of the spindle matrix controls how the fibre assembles or disassembles.

In 1982, Pickett-Heaps and colleagues conducted an experiment where they identified a collar that was visibly seen between the kinetochore and the microtubules. The presence of this collar suggested that there are other proteins surrounding these components that are able to interact with each other as well as with fibers within the

spindle. Further research was conducted and later in *Drosophila melanogaster* additional proteins were discovered that are believed to be part of the spindle matrix, namely Megator, Skeletor, EAST, and Chromator which I will now describe.

E.2.1 Skeletor

Skeletor was discovered in *Drosophila* in 2000 by Walker and colleagues and through antibody staining was found to associate with chromosomes during interphase. As the cell proceeds into prophase, Skeletor redistributes into the cytoplasm. Once in the cytoplasm, Skeletor forms a fusiform spindle structure prior to the formation of a spindle composed of microtubules (Walker et al., 2000). At metaphase, the microtubule-formed spindle and the Skeletor-formed spindle align with each other. When the chromosomes begin to segregate to the poles during anaphase, the skeletor spindle maintains its fusiform structure. When the microtubules were depolymerized using nocodazole, the Skeletor spindle still maintained its shape. This suggests that the matrix provides an ideal structure for providing support and counterbalancing force production (Walker et al., 2000).

E.2.2 Megator

Megator is a matrix protein that was discovered in 2004 by Qi et al., in *Drosophila*. It is the ortholog of the human protein Tpr and has a large coiled-coil domain. Through immunochemistry and cross-immunoprecipitation, it was found that Megator colocalizes with previously discovered matrix proteins in the spindle during mitosis. During interphase, Megator can be found in the nuclear rim and localizes to the space surrounding the chromosomes (Qi et al., 2004). When microtubules were

removed from the cell, the Megator metaphase spindle integrity remained. This implies that the structure does not rely on the polymerization of microtubules in order to remain stable (Qi et al., 2004). The C-terminal domain and N-terminal domain of the protein both have separate functions. When the N-terminal domain was removed from the protein, it was still able to localize both to the nucleus and the mitotic spindle. With only the N-terminal coiled coil domain the protein would stay within the cytoplasm and was still able to form spherical structures. This implies that the C-terminus is required for targeting and localization, while the N-terminus is required for polymer formation and may contribute the structure of the spindle matrix complex (Qi et al., 2004).

E.2.3 EAST

EAST is an additional spindle matrix protein which during interphase is able to form an expandable nuclear endoskeleton (Qi et al., 2005). During interphase, EAST is also able to interact with Megator and localize to the space surrounding the chromosomes. Once mitosis is initiated, EAST relocates with the other spindle proteins mentioned to the cytoplasm where it contributes to the formation of the spindle matrix.

E.2.4 Chromator

Chromator is an additional spindle matrix protein found in *Drosophila*, which directly interacts with the spindle matrix protein Skeletor (Rath et al., 2004). The matrix protein contains a chromodomain and during interphase has been found to associate with chromosomes in a pattern that causes it to overlap with Skeletor. Similar to Megator and Skeletor, during mitosis Chromator detaches from the chromosomes and move to the cytoplasm to form a spindle structure. Like, Megator, the N-terminal and C-

terminal domains have specific roles that contribute to the protein function. The N-terminal domain is responsible for interacting with the chromatin, while the C-terminal domain is important for spindle and nuclear localization (Rath et al., 2004; Yao et al., 2012). When Chromator is depleted in *Drosophila* S2 cells, chromosomes do not segregate properly and the morphology of the microtubule spindle is abnormal.

E.2.5 Additional Proteins in the Spindle Matrix

In addition to the proteins mentioned above, there are many proteins which have been proposed to be a part of the spindle matrix. Examples include Lamin B and Poly (ADP-ribose). Lamin B is a nuclear envelope protein which in the presence of Ran-GTP, is able to form a membrane like structure (Ma et al., 2009). This protein is able to associate with many proteins responsible for spindle assembly including TPX2, NuMA, and Eg5. Due to the relation between Lamin B and spindle assembly proteins, it is believed that this protein plays an important role in spindle assembly (Ma et al., 2009; Tsai et al., 2006). Poly (ADP-ribose) is a large negatively charged macromolecule that has been found to be enriched within in the spindle (Smith, 2001). When the function of Poly (ADP-ribose) was altered the spindle was unable to form properly and hydrolysis of the protein led to the cells inability to form a bipolar spindle (Chang et al. 2004). Additional candidates are discussed in further detail in Johansen and Johansen (2007) as well as Johansen et al., (2011).

E.3 Actin

Many experiments have shown that many of the anomalies that occur in these microtubule based models can be explained by the activity of actin within the spindle. I

will now discuss this actin based model and how it has been shown to contribute to chromosome movements during cell division.

Actin is a globular protein that is approximately 42 kDa and belongs to a multifunctional protein family. Actin has been found to have many different functions within cells such as vesicle transport, cytokinesis and cell signaling (Karp, 1999; Rodriguez et al., 2003). Both the monomeric form and linear polymer of actin are essential in these processes. Like microtubules, actin is a highly dynamic protein that can rapidly change in order to respond to the environment.

There are 3 different isoforms of globular actin which can be found in cellular systems: alpha, beta and gamma (Otterbein, Graceffa & Dominguez, 2001). The beta and gamma isoforms of globular actin are more commonly associated with the cytoskeleton while the alpha form is found in muscle cells forming the contractile unit. Globular actin has two lobes which are separated by a cleft. The region of the protein with the cleft is the site of enzymatic activity where it will bind ATP and Mg2+ and hydrolyze the ATP to ADP. The monomer of actin is active when the cleft is either bound to ATP or ADP but the ATP-bound form is more common in cells (Graceffa & Dominguez, 2003).

When globular actin polymerizes into a long polymer chain it forms a double helix. Similar to microtubules, filamentous actin is also polar in nature as all of the monomers point in the same direction. The minus end of actin is where the ATP binding site is exposed (Bray, 2001). The rate of dissociation at this end is much faster due to the hydrolyzed ATP which destabilizes the polymer. The plus end of actin is the end

where the catalytic site is not exposed and actin monomers which have ATP bound to the cleft are added to this end (Bray, 2001).

The growth of actin filaments is tightly regulated by a number of proteins. Similar to microtubules, a protein complex is required in order to nucleate the actin monomers. The Arp2/3 complex is a large protein unit which contains two subunits that are similar in structure to globular actin (Pollard, 2007). This large protein acts as a nucleation site for actin and is able to make new branches off coexisting actin filaments. Thymosin and profilin are two other proteins which regulate the growth of actin filaments. When profilin binds to globular actin it causes the ADP to be exchanged for an ATP, promoting the addition of globular actin to the plus end (Gunning et al., 2015). Thymosin can bind to globular actin and prevents it from being added to the plus end of the polymer decreasing the rate of growth (Goldschmidt-Clemont et al., 1992).

The plus end and minus end of actin can also be referred to as the barbed and pointed end respectively. The two ends of the actin filament obtained this name from the way actin looks under an electron microscope once decorated with heavy meromyosin (HMM). Ishikawa and colleagues (1969) found that when the fragmented myosin binds to the actin filaments, arrowheads appear along the length of the filament.

Although many suggest that the spindle apparatus is composed of only microtubules and its associated motor proteins, many studies have found this to be untrue. In a study by Forer and Behnke (1972), crane-fly spermatocytes were glycerinated and then treated with heavy meromyosin from rabbit skeletal muscle. When the spermatocytes were visualized under an electron microscope, actin filaments

were seen near chromosomal spindle fibers. This suggests that the spindle is not only composed of microtubules but also includes actin and potentially many other proteins.

E.4 Myosin

I am targeting the motor protein myosin in order to better understand how chromosome movements are accomplished in the spindle. Like actin, this protein has been found to explain many of the inadequacies in the microtubule based models. I will now discuss myosin and provide evidence to show it is a good protein to target when studying chromosome movements.

Myosin is an ATP-dependent motor protein that is commonly known for its role in muscle contraction. Previously, myosin was thought to only be found in muscle cells but have recently been found to be involved in a large number of processes. The structure of myosin across species is widely conserved so much so that a myosin II from the muscle of one organism will be able to bind to an actin polymer in another (Thompson & Langford, 2002). There are 18 different classes of myosin proteins that are defined based on the region of the myosin that does not bind to microfilaments. Each of these classes has a different function in the cell and is used for different types of mobilities. Of these different classes, there are two major categories that myosin can be organized into. The first are the conventional myosin II proteins which are involved in muscle contractions. The second are myosin proteins that are not involved in muscle contraction and are considered unconventional (Bray, 2001; Sellers, 2000).

Myosin is composed of 3 main domains that are found in most of the isoforms. The head domain is the region that is able to bind to filamentous actin (Warrack &

Spudich, 1987). This same domain also has catalytic activity which allows the myosin to hydrolyze ATP. This enzymatic activity is what gives the myosin the ability to walk along filaments in a plus-end directed manner. All myosin proteins are plus end directed except for myosin VI which travels along the filament in a minus end-directed manner. The neck domain connects the head domain with the subsequent domain (Warrack & Spudich, 1987). It is also responsible for acting as a lever allowing for force production when the ATP is hydrolyzed. This domain also contains regions that allow for the attachment of light chains. These light chains are distinct from the rest of the protein structure and are usually regulatory in nature. The last domain is the tail domain. This domain is unique for every class of myosin and is used to bind different types of cargo (Warrack & Spudich, 1987). The tail domain may also have a role in regulating the motor activity of the myosin.

Myosin is able to produce force through a power stroke that is generated through the hydrolysis of ATP (Tyska & Warshaw, 2002). When the myosin head hydrolyzes ATP it is able to bind to the actin filament. The ADP and inorganic phosphate complex stay bound to the myosin head. The loss of the inorganic phosphate induces a conformational change in the myosin causing the stalk to bend, pulling the actin filament. This is known as the power stroke. Once the ADP is removed and an ATP is added, the myosin head is able to release the actin filament. This process continues until signals for contraction and mobility cease.

The models mentioned above that allow for chromosomes to move during anaphase ignore many proteins and only concentrate on the presence of microtubules and its motor proteins. Both actin and myosin have been found in many different cell

types to be associated with the spindle and play a large role in chromosome movement. Fujiwara and Pollard (1976; 1978) were able to detect myosin in the spindles of HeLa cells when the cells were fixed and observed under fluorescence microscopy. When fluorescently labeled myosin was introduced into PtK cells and Drosophila embryos, myosin was also found in the spindles (Royou et al., 2002; Sanger et al. 1989).

Myosin has not only been located in the spindle of different cells but is also important for chromosome movement. When myosin activity was blocked in rat kidney cells, anaphase in the cells was unable to occur (Komatsu et al., 2000). Myosin was also found to be important not only in chromosome movements but also in spindle formation. When myosin was inhibited in NRK cells and sea urchin zygotes, spindles were unable to form or microtubule organization was altered (Fishkind et al., 1991; Mabuchi et al., 1990).

E.4.1 Myosin Regulation

Myosin is composed of two non-muscle myosin II heavy chains (NMHCs), 2 essential light chains and 2 light chains that are regulatory in nature. Myosin activity is regulated through the phosphorylation and dephosphorylation of the myosin regulatory light chains (MRLC) (Somlyo & Somlyo, 2003). The phosphorylation of the myosin regulatory light chain is controlled either by hormonal or neuronal means. When the cell is activated by either one of these mechanisms calcium is released into the cell in high concentrations. The excess calcium in the cell is then able to bind to calmodulin (CaM). When four calcium molecules bind to calmodulin a conformational change is induced (Cremo & Hartshorne, 2008; Bray, 2001). The conformational change causes

hydrophobic methyl groups to become exposed, giving it a higher affinity to target proteins. One of the target proteins that activated calmodulin can bind to is myosin light chain kinase (MLCK) which activates it. Once active, the kinase is able to phosphorylate the myosin regulatory light chain, activating myosin (Cremo & Hartshorne, 2008; Bray, 2001).

The regulatory light chain can be phosphorylated at either one or two residues. The preferred residue to phosphorylate is serine 19 (Ikebe & Hartshorne, 1985; Tan et al., 1992). When the myosin regulatory light chain is monophosphorylated, two things happen. The ATPase activity of the myosin head become activated due to a conformational change. When the regulatory light chain is not phosphorylated it is folded so that it interacts with the head of the myosin. Upon phosphorylation, the light chain changes shape so that ATPase activity is activated (Trybus, 1991). The phosphorylation of the regulatory light chain also increases the stability of the myosin filaments and encourages myosin aggregation and assembly. Once these two events have occurred myosin cross bridges can act as a motor and contribute to force production and contraction. The second site that can be phosphorylated by a kinase is threonine 18 on the myosin regulatory light chain. This site is less common and less preferred compared to serine 19 (Tan et al., 1992). When diphosphorylation occurs the same two events occur but more significantly (Ikebe & Hartshorne, 1985; Ikebe et al., 1988). Although both events occur more significantly it has been found to have little effect on the velocity of myosin in smooth muscle (Umemoto et al., 1989).

If there is a mutation with the myosin regulatory light chain, myosin activity becomes diminished. In a Drosophila mutant, *Spaghetti squash*, the myosin regulatory

light chain is altered in a way that it cannot be phosphorylated. Studies found that cells were not able to complete cell division as cytokinesis was greatly impacted (Karess et al., 1991).

The myosin regulatory light chain can be either monophosphorylated or diphosphorylated but the relative abundance and distribution of both are strikingly different. For monophosphorylated light chains, the distribution and abundance are similar to that of myosin II (Matsumura et al., 1998; Sakurada et al., 1998). Diphosphorylated light chains, however, tend to be more localized and only found within certain regions. This form tends to be found more commonly in smooth muscle and the concentrations can change very quickly within the cell (Sakurada et al., 1998; Uchimura et al., 2002).

The myosin regulatory light chain can be phosphorylated by a number of different kinases, namely Myosin light-chain kinase (MLCK), Rho-associated protein kinase (ROCK), citron kinase, myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), zipper-interacting protein kinase (ZIP kinase) and integrin-linked kinase (ILK) (Kamm & Stull, 2001; Amano et al., 1996; Yamashiro et al., 2003) . All of these kinases are able to phosphorylate serine 19 and threonine 18 on the myosin regulatory light chain. Unlike phosphorylation, dephosphorylation of the myosin regulatory light chain can only be done by a single phosphatase. Myosin light chain phosphatase has an MYPT1 subunit that binds to myosin II allowing for phosphatase activity (Hartshorne et al., 2004). The activity of this subunit is controlled through phosphorylation by ROCK (Kimura et al., 1996).

One of the drugs used for this thesis was 2,3-butanedione 2-monoxime (BDM), which directly inhibits the activity of myosin. BDM is an uncompetitive inhibitor, binding to myosin on a different region from the ATP binding site (Herrmann et al., 1992). This inhibitor has been found to effect skeletal myosin and binds to the myosin once the ATP has bound to the myosin head (Higuchi & Takemori, 1989). The ability of myosin to inhibit skeletal myosin has been debated recently because the ATPase activity of myosin was found to not be affected (Forer & Fabian, 2005). Studies have come out that determined that BDM does not inhibit ATPase activity but rather alters mobility (Krementsov et al., 2004). When BDM binds to myosin it stabilizes the ADP-Pi complex and as a result prevents the power stroke from occurring (Forer & Fabian, 2005).

E.4.1.1 Myosin Light-chain Kinase (MLCK)

Myosin light chain kinase is a specific serine/threonine protein kinase that phosphorylates the myosin regulatory light chain (Gao et al., 2001). There are four different known isoforms of myosin light chain kinase. MYLK is found in smooth muscle, MYLK2 in skeletal muscle, MYLK3 on cardiac muscle and MYLK4 which is a novel isoform (Reviewed in Manning et al., 2002). The activity of myosin light chain kinase is itself regulated by phosphorylation. cAMP-dependent protein kinase (PKA) is an example of a protein kinase that can phosphorylate myosin light chain kinase at two different sites. This only occurs however when the concentration of active calmodulin is low. As calcium is sequestered from the cell and active calmodulin decreases the concentration of phosphorylated myosin light chain kinase increases (Lukas et al., 1986).

Myosin light chain kinase is able to bind to two different structures. It can bind the actin filaments and globular actin through the N-terminal sequence. The C-terminal sequence is able to bind to the serine 19 residue on the myosin regulatory light chain on the myosin (Cremo &Hartshorne, 2008). The role of the enzyme has been extensively studied with respect to its role in mitosis and meiosis.

In a study by Silverman-Gavrila and Forer (2001), ML-7 was added to crane-fly spermatocytes and chromosomes were unable to properly attach to the spindle. A second effect that was observed was that chromosome movement during anaphase was also altered. Similar results were found by Sheykhani, Shirodkar, and Forer (2013) when adding myosin inhibitors to crane-fly spermatocytes. In addition to drug treatments, immunofluorescent staining found that the levels of monophosphorylated and diphosphorylated myosin decreased, indicating that myosin light chain kinase was being targeted by the drug.

Other than pharmacological studies, genetic studies have also been done in order to determine the role of myosin light chain kinase during mitosis. Komatsu and colleagues (2000) overexpressed a myosin regulatory light chain that was not able to be phosphorylated. Results indicated that this mutation prevented the onset of anaphase in rat cells. Additional studies were also done in HeLa cells that had a genetic disruption in the myosin light chain kinase. Dylyaninova and colleagues (2004) found that chromosomes were unable to align properly on the metaphase plate and microtubule defects were present.

The drug that I used in order to inhibit the function of myosin light chain kinase during prometaphase in Mesostoma primary spermatocytes is 1-(5-Iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine hydrochloride (ML-7), so I will explain it in more detail. ML-7 is a myosin light chain kinase inhibitor that acts through reversible competitive inhibition of the ATP binding site. It is able to inhibit both calcium calmodulin dependent and independent myosin light chain kinase. Other kinase inhibitors such as ML-9 can be used but ML-7 is 10x more potent (Bain et al., 2003). ML-7 not only targets MLCK but can also inhibit PKA and PKC, so I also used 5-(2-Methylpiperazin-1-yl)sulfonylisoquinoline dihydrochloride (H-7) in order to determine if the results obtained were from MLCK inhibition. H-7 is a kinase inhibitor but affects the activity of PKA and PKC at much lower concentrations compared to ML-7, while it requires approximately 97 uM in order to affect MLCK activity (Saitoh et al., 1987; Quick et al., 1992; Silverman-Gavrila & Forer, 2001). If the addition of H-7 does not have any effect on chromosome movements, this suggests that chromosome movement perturbations during ML-7 are from myosin light chain kinase inhibition.

E.4.1.2 Rho-associated Protein Kinase (ROCK)

Rho-associated protein kinase is a serine-threonine specific protein belonging to the PKA/PKG/PKC family. It is commonly known for its role in manipulating the cytoskeleton in order to alter the shape and movement of cells (Narumiya, 1996). The protein is approximately 158 kDa and is a major effector of RhoA, a GTPase. The kinase contains 2 different domains, a kinase domain and a Pleckstrin homology (PH) domain which is responsible for activity. When RhoA-GTP is not present the PH domain is folded and inhibits the function of the kinase (Hahmann & Schroeter, 2010). Two

isoforms of rho-associated protein kinase have been identified in mice. ROCK1 is expressed in the testis, liver, and kidney, whereas ROCK2 is expressed in the heart and brain (Nakagawa et al., 1996).

Rho-GTP activates rho-associated protein kinase which is then able to increase myosin activity and actin filament organization through two different mechanisms. The first mechanism is through the phosphorylation of the myosin regulatory light chain. The second mechanism is through the phosphorylation of the site on myosin light chain phosphatase increasing contraction (Amano et al., 1996; Ishizaki et al., 2000; Kimura et al., 1996).

ROCK has also been found to play a role during the cell cycle. The kinase has been found to ensure that centrioles do not separate prematurely during the G1 phase (Hahmann & Schroeter, 2010). Additional studies have found the importance of ROCK during cell division. Studies have found that when ROCK is inhibited in *Xenopus* and *Caenorhabditis elegans* embryos, cleavage furrow contraction is inhibited, and cytokinesis is unable to go to completion (Drechsel et al., 1996; Piekny & Mains, 2002). Y-27632 is a ROCK inhibitor that has been used extensively in studies to determine the effects on cells during mitosis and meiosis. When crane-fly spermatocytes were treated with Y-27632 during anaphase, chromosome movements were either stopped or slowed (Fabian & Forer, 2005; Fabian et al., 2007). When the ROCK inhibitor was added the PtK2 cells spindle organization was altered as well as the positioning of the centrosomes (Rosenblatt et al., 2004). The above studies all indicate the importance of Rho-associated protein kinase on chromosomal movements and spindle formation through its action on myosin.

In order to inhibit the activity of ROCK, the drug I used was (R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y-27632), a pyridine derivative. Y-27632 is a specific Rho-kinase inhibitor. It's mode of action is through inhibiting calcium sensitization, preventing myosin activity (Uehata et al., 1997).Y-27632 is also able to inhibit cAMP-dependent protein kinase, PAK, PKC, and MLCK, but it is greater than 200 fold times more sensitive for ROCK.

E.4.1.3 Integrin-linked Kinase (ILK)

Integrin-linked Kinase (ILK) was first identified in 1996 and functions as a serine/threonine kinase (Hannigan et al., 1996; Li, Zhang & Wu, 1999). ILK has many roles in the cell including cell proliferation, angiogenesis and microtubule dynamics (Hannigan, Troussard & Dedhar, 2005; Lim et al., 2013). ILK can also interact with many focal adhesions proteins such as paxillin, allowing it to regulate actin cytoskeletal dynamics (Legate et al., 2006).

In a previous study, ILK was found to bind to tubulin and functions to organize the mitotic spindle. Inhibition of ILK using QLT-0267, a competitive inhibitor, lead to mitotic spindle disorganization, effecting microtubule dynamics (Fielding et al., 2008). When the activity of ILK was overexpressed in HeLa cells, the length of mitosis decreased and the spindle was less sensitive to microtubule-targeted drugs (Lim et al., 2012).

E.4.1.4 Citron Kinase (CK)

Citron Kinase (CK) is a protein that has a similar domain to the Rho-activated kinase family and Rho family GTPase downstream effectors (Di Cunto et al., 1998;

Madaule et al., 1998). Citron kinase is approximately 183 kDa with 4 distinctive domains. It contains a zinc finger, PH domain, rho/rac binding site and a long coiled region containing 4 leucine zippers (Madaule et al., 1995). CK is able to phosphorylate the myosin regulatory light chain at both Serine 19 and Threonine 18, similar to other Rho-activated kinase, but does not have an inhibitory effect on protein phosphatase 1 (Cremo & Hartshorne, 2008).

When ROCK is inhibited in PtK cells by Y-27632, citron kinase still maintains its ability to bi-phosphorylate and maintain stress fibers (Yamashiro et al., 2003). Citron kinase has also been found to play a major role in forming and maintaining the cleavage furrow during cytokinesis. Madaule and colleagues (1998), conducted experiments where citron kinase was overexpressed or mutated in HeLa cells. Results found that the overexpression of citron kinase caused the cells to become multinucleate. When a kinase active mutant of citron kinase is introduced, the contractile ring ingresses in an abnormal fashion, indicating that citron kinase regulates cytokinesis after Rho kinases. Naim and colleagues (2004) conducted RNA interference mutation experiments targeting citron kinase. Cytokinesis was not able to be completed properly in both drosophila spermatocytes and neuroblasts. In addition to the incompletion of cytokinesis, filamentous actin became disorganized suggesting that normal functioning CK is required for abscission in cytokinesis.

E.4.1.5 Myosin Regulatory Light Chain Dephosphorylation and Protein Phosphatase 1

When the myosin regulatory light chain is dephosphorylated this leads to the loss of catalytic ATPase ability of the myosin head. The protein that is responsible for the inactivation of myosin catalytic activity is in the class protein phosphatase 1 (PP1). Within this type, there are two different dependent manners in which the protein can function. The phosphate can either be an aspirate-based phosphatase or it can be a metal-dependent phosphatase. The function of protein phosphatase 1 has been extensively studied and has been found to play an important role in muscle contraction, apoptosis, mitosis and RNA splicing (Tournebize et al., 1997).

There are many different types of phosphatases in the cell and the types are organized based on the catalytic subunit present (Hartshorne et al., 2004). The catalytic subunit is able to form complexes with other regulatory units on the protein. The protein is composed of an additional 3 subunits. There is a regulatory and small subunit, as well as MYPT1 which was previously mentioned. This subunit is responsible for targeting and binding to myosin in order to regulate activity (Cremo & Hartshorne, 2008).

The function of PP1 can be regulated by a number of different compounds. As mentioned previously, rho-kinase is able to phosphorylate the MYPT1 subunit at both threonine 696 and 853, preventing PP1 from binding and targeting myosin (Ito et al., 2004). There are also a variety of naturally occurring PP1 inhibitors including okadaic acid, tautomycin, microcystin and Calyculin A

For my thesis, the effects of Calyculin A and okadaic acid were studied on *Mesostoma* primary spermatocytes, so I will explain them in more detail. Calyculin A is a naturally occurring compound isolated from the sponge *Discodermia calyx* (Suganuma et al., 1990). The compound is composed of a long fatty acid chain, esterified by phosphoric acid and has two gamma-amino acids. Calyculin A inhibits Ser/Thr protein phosphatase 1 and 2A and through its action induces contraction in smooth muscle (Ishihara et al. 1989). In a study by Ashizawa, Magome, and Tsuzuki (1995), Calyculin A was able to activate sperm motility in fowl spermatozoa even after they had become immobile, but was only able to accomplish this in the presence of free calcium. In additional studies by Sakurada et al. (1988), smooth muscle cells were treated with Calyculin A and the bi-phosphorylation of myosin regulatory light chain was observed.

Additional studies were conducted by Fabian et al. (2007). In order to determine the effects of protein phosphatase 1 suppression on chromosome movement, calyculin A was added to crane fly spermatocytes shortly after anaphase began, after which chromosomes moved to the pole with a higher velocity with a few chromosomes moving backward. Due to the function of Calyculin A, the increase in chromosome velocity to the pole is thought to be a result of hyper phosphorylation. The hyper phosphorylation of the myosin regulatory light chain would then lead to the hyperactivity of myosin.

Calyculin A can inhibit PP1A and PP2A. To test whether the increase in velocity was due to the inhibition of PP1A or PP2A, okadaic acid was used. Okadaic acid is a toxin that is produced mainly by dinoflagellates but can also be found in shellfish and sponges (Reguera et al., 2014). Okadaic acid is known to strongly inhibit protein

phosphatases especially those that are Serine/threonine phosphatases (Dounay & Forsyth, 2002). Although okadaic acid can inhibit both PP1 and PP2A, PP1 can only be targeted at higher concentrations, so it can be used as a control to determine if drug treatments by calyculin A are a result of PP1 or PP2A. In a study by Cohen, Klumpp and Schelling (1989), it was found that PP2A is completely inhibited at 1-2 nM okadaic acid while PP1 inhibition requires at least 1 μM. When Fabian and colleagues (2007) treated crane fly spermatocytes with okadaic acid, no increase in poleward velocity of chromosomes was observed indicating that the effects were due to the inhibition of PP1 and not PP2A.

E.5 Models for Actin and Myosin based Movement

As previously mentioned, there are mainly models explaining how microtubules and its motor proteins contribute to chromosome movements during mitosis and meiosis. Many experiments have been conducted that show the involvement of actin and myosin in this movement and how previous models are inadequate. There are many anomalies that occur in cells that cannot be explained solely by the presence of microtubules and the associated motor proteins.

Silverman-Gavrila and Forer (2000), wanted to determine whether microtubule flux relied only on microtubule dynamics or if actin and myosin were involved in the mechanism. When the microtubules are stained for acetylated tubulin, a gap in fluorescence would represent flux in the microtubule. When actin inhibitors including cytochalasin D, latrunculin B, and swinholide were used to treat crane-fly spermatocytes the gap in fluorescence disappeared. Similar results were seen when the spermatocytes

were treated with BDM, a myosin inhibitor. The disappearance of the gap indicates that tubulin subunit are staying in place on the microtubule for a longer time. The loss in the gap also indicated the new tubulin monomers are not being added at the kinetochore and therefore flux has been inhibited.

Forer and colleagues (2007) conducted UV microbeam irradiation experiments in crane-fly spermatocytes and inhibited actin and myosin. Kinetochore fibres were cut during metaphase in crane-fly spermatocytes. According to the Pac-Man model, the microtubule stub attached to the kinetochore should shorten as a result of depolymerisation at the plus end. When actin targeting drugs were used such as those mentioned in the previous study, the kinetochore fibre stubs were not able to increase in length through flux, nor were they able to shorten through the Pac-Man model. Similar results were obtained where the myosin inhibitor, BDM, was used to treat crane fly spermatocytes (Forer et al., 2007).

Additionally, studies have also been conducted in order to determine the role of both actin and myosin in relation to chromosome movement as well as chromosome spindle attachment. In a study by Silverman-Gavrila and Forer (2001), crane-fly spermatocytes were treated with anti-myosin drugs in order to determine the effects on chromosome movement and cytokinesis. When BDM was added to spermatocytes during anaphase, chromosome movement to the pole slowed down, stopped or began to move backwards. To confirm myosin was being inhibited ML-7 was also added to spermatocytes during prometaphase. Addition of the drug at this stage prevented chromosomes from being able to attach to the spindle (Silverman-Gavrila & Forer, 2001). BDM and ML-7 also altered contraction of the contractile ring during cytokinesis.

BDM only had an effect on the contractile ring once it was formed causing ingression to stall or relax. ML-7 had similar results but effected furrow ingression at all stages.

Fabian and Forer (2007) used locust spermatocytes in order to further identify possible roles of actin and myosin in relation to chromosome movements during anaphase. When actin inhibitors including latrunculin B and cytochalasin D were used, chromosome movement towards the poles halted during anaphase. Chromosomes also slowed with a few accelerating. The addition of myosin drugs also yielded similar results. The addition of BDM and Y-27632 caused the majority of chromosomes to slow or come to a complete stop during segregation. ML-7 had the same results as when actin inhibitors were added with chromosomes accelerating, slowing or stopping (Fabian & Forer, 2007).

F. Methods for Studying Chromosome Movement

In my thesis, I used pharmacological studies in order to determine the effect of different inhibitors and activators on chromosome movement in *Mesostoma* primary spermatocytes. I did not study the effects of drugs on microtubules, matrix proteins, or actin and instead focused on the role of myosin in the spindle. Various drugs were used that either directly inhibited myosin activity or altered myosin phosphorylation by affecting upstream proteins. In addition to inhibiting the activity of myosin, I also used drugs in order to increase myosin activity to determine the effects on prometaphase oscillations. My thesis, with support from previous studies suggests that myosin is a major force contributor, and contributes to proper chromosome movements. My thesis also suggests that myosin is not the only major protein involved and works in

conjunction with an array of proteins that interact with the spindle to allow proper movement and segregation

Introduction

Chromosomal movements during cell division have been extensively studied for years. Despite extensive research, the cytoskeletal components that are involved in chromosomal movements have yet to be determined. Current models suggest that microtubules and their motor proteins within the spindle are the sole force producers (Gorbsky et a., 1988; Mitchinson, 2005; Nicklas, 1989, Rogers et al, 2005). Others, however, have suggested that other cytoskeletal components contribute to force production allowing for chromosomal movements such as actin (Forer & Pickett-Heaps, 1988; Silverman-Gavrila, 2000; Fabian & Forer, 2005; Snyder et al., 2010), myosin (Fabian & Forer, 2005; Fabian et al., 2007; Sheykhani et al., 2013; Silverman-Gavrila and Forer, 2000) and spindle matrix proteins (Qi et al., 2004; Qi et al., 2005; Rath et al., 2004; Walker et al., 2000; Yao et al., 2012). My thesis describes experiments that depict the role of spindle myosin in bivalent oscillations during prometaphase in primary spermatocytes.

Myosin interacts with a number of different cytoskeletal components including spindle microtubules (Rodriguez et al., 2003). In crane-fly spermatocytes myosin has been localized to the spindle fibres during cell division (Fabian & Forer, 2005; Fabian et al., 2007; Sheykhani et al., 2013; Silverman-Gavrila & Forer, 2001). Pharmacological experiments provide evidence that myosin is involved in poleward chromosomal movements and microtubule flux (Silverman-Gavrila & Forer, 2001; Fabian & Forer, 2005; Fabian et al., 2007). When myosin activity was inhibited in PtK cells, spindle organization was disrupted and centrosomes were unable to align properly (Rosenblatt

et al., 2004). In locust spermatocytes, myosin was inhibited and the movements of chromosomes during anaphase slowed or stopped and in some cases accelerated (Fabian & Forer, 2007).

Myosin activity is regulated through a number of different pathways which alter the phosphorylation of the myosin regulatory light chain (Komatsu et al., 2000; Seller, 1991; Trybus, 1991). The myosin regulatory light chain can be phosphorylated on threonine 18 and serine 19 which is the preferred site (Ikebe & Hartshorne, 1985; Tan et al., 1992). Multiple pathways can contribute to myosin regulatory light chain phosphorylation including myosin light chain kinase, Rho-associated protein kinase, and several others. These different kinases can either monophosphorylate or diphosphorylate the myosin regulatory light chain in order to activate myosin (Amano et al., 1996; Cremo & Hartshorne, 2008; Sakurada et al., 1998). In order to deactivate myosin, only a single dephosphorylation pathway is involved. Protein phosphatase 1 regulates myosin activity through the dephosphorylation of the myosin regulatory light chain, inhibiting myosin catalytic activity.

In order to study the role of myosin phosphorylation in chromosomal movements during cell division, different phosphorylation inhibitors have been used. Various inhibition studies have shown defects in cell division once myosin activity has been inhibited. For example, Fabian and Forer (2007) treated crane-fly spermatocytes with Y-27632, which inhibits the activity of Rho-kinase after which chromosome movement during anaphase slowed or stopped. In addition to Y-27632, spindle myosin activity can be inhibited by treating cells with ML-7. ML-7 inhibits the activity of myosin by blocking myosin light chain kinase. When crane-fly spermatocytes were treated with ML-7,

chromosome movements during anaphase stopped or slowed with a few accelerating (Fabian & Forer, 2007). They concluded that Rho-kinase and myosin light chain kinase are some of the many pathways that can activate spindle myosin to allow for proper chromosomal movements. The activity of myosin can be enhanced by treating cells with calyculin A. Calyculin A inhibits the activity of protein phosphatase 1, and as a result induces hyperphosphorylation of the myosin regulatory light chain (Ishihara et al., 1989; Sakurada et al., 1988). Shortly after the start of anaphase, crane-fly spermatocytes were treated with calyculin A, after which the chromosomes moved to the pole with a higher velocity (Fabian et al., 2007).

Sheykhani et al. (2013) used both ML-7 and Y-27632 to treat crane-fly spermatocytes during anaphase. Once the movement of the chromosomes had slowed or stopped, they then challenged the drug by adding calyculin A. The addition of calyculin A to the cell caused the movement of chromosomes to speed up. These results indicate that in addition to Rho-kinase and myosin light chain kinase there are additional pathways that contribute to spindle myosin activity. Silverman-Gavrila and Forer (2001) treated crane-fly spermatocytes with BDM during anaphase. BDM inhibits the activity of myosin by stabilizing the ADP and inorganic phosphate complex, thus preventing the power stroke. In the presence of BDM, chromosome movements during anaphase stopped or slowed with a small number speeding up. From these results, it was concluded that although myosin is involved in the force production that allows chromosome movements to occur during cell division, it is not the only force producer.

In my thesis, I investigate the role of myosin phosphorylation on bivalent oscillations in *Mesostoma ehrenbergii* primary spermatocytes. *Mesostoma* primary

spermatocytes have 3 large bivalents that oscillate regularly for 1-2 hours during prometaphase before the onset of anaphase. If spindle myosin in *Mesostoma* primary spermetocytes acts like myosin in previous studies I expect that spindle myosin provides force that contributes to bivalent oscillations and that there are many phosphorylation pathways and mechanisms. I predict that when myosin activity is inhibited, bivalent oscillation velocities will decrease. When the activity of myosin is enhanced, I expect chromosome velocities to increase. I my experiments, I added different inhibitors separately and together during prometaphase and followed bivalent oscillations.

Materials and Methods

Rearing *M. ehrenbergii*

M. ehrenbergii are reared in the lab as described by Hoang et al. (2013). Briefly, the stock was started by hatching dormant eggs that were obtained from worms collected from Lake Rondeau, Ontario by Hebert and Beaton (1990). The eggs are hatched under anaerobic conditions by sealing them in water-filled glass jars that contain algae. A few days later the jars are moved to light conditions and the babies are fed brine shrimp. Animals from these eggs are considered the first generation and subsequent generations will continue if S eggs (viviparous embryos) develop. The jars containing *M. ehrenbergii* are stored in an incubator to control for temperature and light (Environmental Growth Chambers Model: TC-1). The *M. ehrenbergii* are fed with brine shrimp on a daily basis and are sometimes fed *Daphnia* instead. *M. ehrenbergii* are also stored in two rectangular tanks at room temperature by the window.

Dissection and preparations of live *M. ehrenbergii* primary spermatocytes

M. ehrenbergii to dissect are chosen based on their maturity. The animals are chosen to be dissected as soon as their ovaries turn white, which occurs at about 2 weeks. The worms develop D-eggs at about 3 weeks and usually have well developed testes. *M. ehrenbergii* that have 2-3 diapausing eggs and translucent cloud-like testes are chosen for dissection since these animals seem to have the most primary spermatocytes. To prepare the animal for dissection they are rinsed 3 times in

Mesostoma Ringer's solution (61mM NaCl, 2.3mM KCl, 0.7mM CaCl2, and 1.4mM phosphate buffer [KH_2PO_4 & $Na_2HPO_47H_2O$], pH6.8) once each in 3 separate Petri dishes. The worm is then pipetted into Ringer's held in a well made from vacuum grease on a glass slide. Excess Ringer's solution is sucked out from the well in order to limit the movement of the worm. The testes are removed using a pulled 10 µL needle (VWR International) connected to a Tygon tube with a mouthpiece. The needle is then inserted into the worms body and the testes are sucked up and placed onto a flamed coverslip. The testes are then mixed with 20 mg/mL fibrinogen (Calbiochem, La Jolla, CA.) in *Mesostoma* Ringers. The cells are then spread and flattened before adding an equal amount of thrombin (Sigma Chemical Co., St. Louis, MO), 50 U/mL, to form a fibrin clot. The coverslips are then sealed onto perfusion chamber slides using valap (vaseline: lanolin: paraffin, 1:1:1).

Drug Treatments

Prior to the addition of drug *M. ehrenbergii* primary spermatocytes are perfused with *Mesostoma* Ringers and normal movement is recorded. Experiments are performed at room temperature which is approximately 23°C. The following inhibitors were used at the final concentrations specified below: 200 µM Y-27632 ((R)-(+)-trans-N-(4-pyridyl)-4-(laminoethyl)- cyclohexanecarboxamide (LC Laboratories), 10, 15 and 20 µM ML-7 (1~(5-iodonaphthalene-l-sulfonyl)-lH-hexahydro-l,4-diazepine hydrochloride) (Toronto Research Chemicals), 1, 2.5, 5 and 10 nM Calyculin A (LC Laboratories), 5 and 10 nM Okadaic acid (LC laboratories), 20 mM 2,3-butanedione 2-monoxime (BDM) (Sigma) and 20 µM H-7 (Sigma). Stock solutions of all drugs excluding BDM are dissolved in DMSO and stored at 1000 times the desired concentration at -40°C.

Measured volumes are thawed and diluted with Mesostoma Ringers to 1:1000 of the original concentration to avoid adverse effects of DMSO on the cells. BDM stocks are made with *Mesostoma* Ringer's and diluted to 1:1000 before adding to the cells. As a control, *Mesostoma* primary spermatocytes were treated with 0.1% DSMO, and no effects were observed.

Phase-contrast Microscopy and measurements

Live cells, both control, and treated cells were observed with a 100X oil immersion Nikon objective (NA = 1.3) using phase-contrast microscopy. Images were recorded in real time on DVD discs and were later time lapsed to .avi files using Virtual Dub freeware (www.virtualdub.org). Kinetochore distance from the edge of the cell was measured using Winimage [program developed in the lab] (Wong & Forer, 2003). Data was then plotted using a commercial program called SlideWrite. Once the data was plotted velocities, amplitude and period were calculated for each kinetochore.

Statistical Analysis

Two-tail Student's t-tests were completed in order to determine if the velocities, amplitudes and periods before and after drug treatment were significantly different. The differences in these parameters were calculated as percentages as seen in table 5. The percentages were first transformed into proportions and then the $\arcsin^{-1}$ was calculated using Excel. The two-tail student's t-tests were then completed using Excel with a confidence interval of 95%.

Results

Control Cells

Mesostoma ehrenbergii primary spermatocytes have three large bivalents and 4 univalents. During prometaphase, the three large bivalents oscillate back and forth as seen in figure 1. The oscillations occur for 1-2 hours before the onset of anaphase. My goal is to determine if drugs that target myosin phosphorylation effect normal oscillations. From the oscillations, I can measure the velocity of kinetochores towards and away from the pole, amplitude and period. The average values for the control cells I used are seen in table 1. The velocity of the kinetochore is faster when moving towards the pole compared to away from the pole.

Table 1. Comparison of oscillation parameter data obtained by Fuge (1987, 1989), Ferraro-Gideon, Hoang, and Forer (2014) and my data for Mesostoma primary spermatocyte control cells.

	Mesostoma Primary Spermatocyte Control Cells		
	Fuge (1987, 1989)	**Ferraro-Gideon, Hoang, and Forer (2014)**	**My data***
Average Velocity	8 – 10 µm/min	*To pole:* 6.24 µm/min *Away from pole*: 5.23 µm/min	*To pole:* 7.66 ± 2 (SD) µm/min *Away from pole:* 6.44 ± 1.38 (SD) µm/min
Average Amplitude	5 – 7 µm	4 µm	3.58 µm ± 0.55 (SD)
Average Period	1 minute 40 seconds	1 minute 32.5 seconds	1 minute 14 seconds
*n = 15 cells, 30 kinetochores, $p \leq 0.01$ for difference movement towards and away from pole			

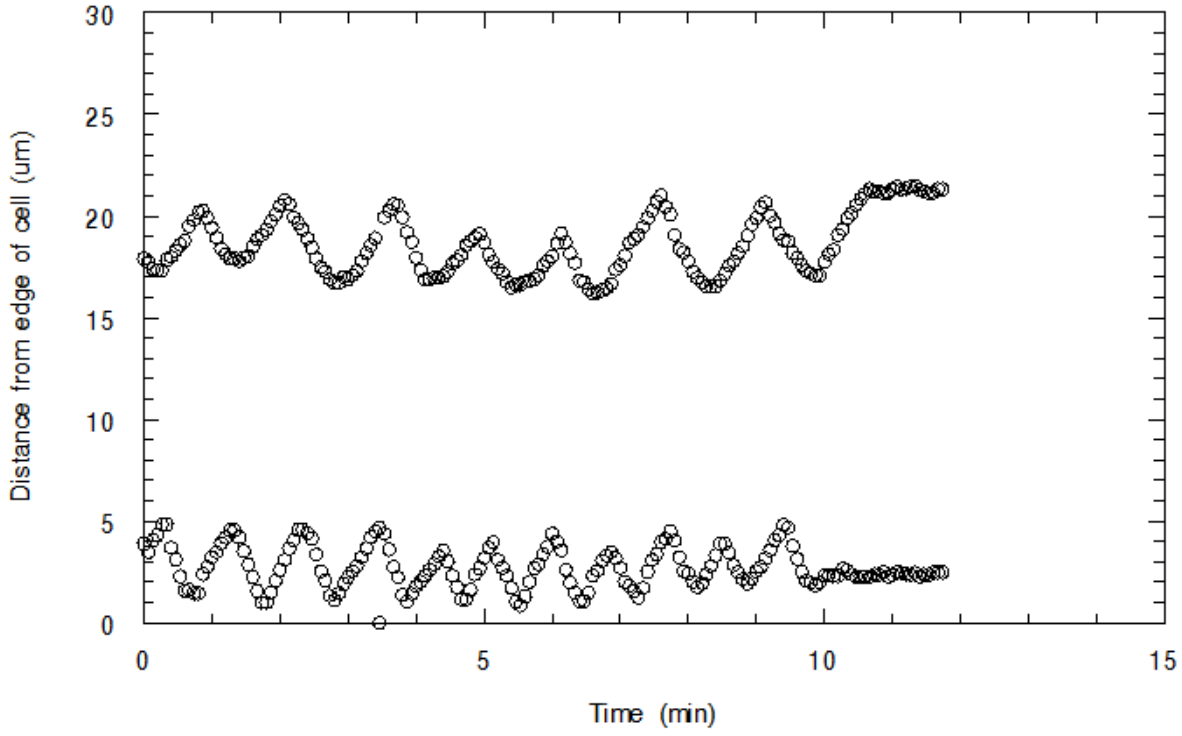


Figure 1. Single bivalent oscillation in a *Mesostoma ehrenbergii* primary spermatocyte control cell. In the first 3 minutes, kinetochore movement is in phase. Both of the kinetochores move away or towards the poles at the same time. At 3 minutes the top bivalent lingers in the position away from the pole causing the kinetochores to switch to move out of phase with each other. After this point, one kinetochore moves towards one pole and the other moves away. Anaphase occurred at about 10 minutes and completed within 1 minute.

When both sister kinetochores are able to be measured the length of the bivalent can be determined. Depending on whether the sister kinetochores are moving in or out of sync with each other, the bivalent can either be stretched or compressed. When the bivalent is compressed the arms of the bivalents move in closer towards each other. In order to get the true length of the bivalent, the length was only measured when the sister kinetochores were not in sync with each other allowing for the length to be more accurate. When the kinetochores are not in sync, one kinetochore will move towards the pole while the other moves away from the pole. The values obtained are seen in table 2.

The length of the cell stays consistent throughout prometaphase and the average length observed is shown in table 2. The length of the cell only changes significantly when the spermatocytes begin to die or after anaphase. The length of the cell decreases as the cell dies and the whole cell starts to round. When the cell completes anaphase the length of the cell increases as cytokinesis begins.

Table 2. Comparison of primary spermatocytes cell and bivalent length obtained by Fuge (1987, 1989), Ferraro-Gideon, Hoang, and Forer (2014) and my data in control cells.

	Mesostoma Primary Spermatocyte Control Cells		
	Fuge (1987, 1989)	**Ferraro-Gideon, Hoang, and Forer (2014)**	**My data**
Average Cell Length	40 µm	30 µm	26. 56 µm ± 1.97 (SD)*
Average Bivalent Length	27 – 40 µm	20 – 25 µm	15.66 µm ± 1.91 (SD)**
*n= 9 cells, **n= 15 bivalents			

During the start of prometaphase, a precocious cleavage furrow forms and slowly ingresses as the cell gets closer to the start of anaphase. The furrow also moves along the length of the cell in response to chromosome movements in the cell. During prometaphase, univalents can switch between poles until the start of anaphase (figure 2). By the start of anaphase, a single metacentric and acrocentric univalent will be at each pole. If a univalent moves, creating an uneven distribution in the chromosomes, the furrow will move towards the pole with fewer chromosomes as seen in figure 2. The kinetochores of bivalents can also switch poles so that both kinetochores are facing the same pole or so kinetochores switch poles (figure 3). The average values for the control cells I used are in table 3.

Table 3. Comparison of primary spermatocytes cell and bivalent length obtained by Fuge (1987, 1989), Ferraro-Gideon, Hoang, and Forer (2014) and my data in control cells.

	Mesostoma Primary Spermatocyte Control Cells	
	Ferraro-Gideon, Hoang, and Forer (2014)	**My data**
Univalent Excursions	1 every 21.5 minutes	1 every 25 minutes*
Bivalent Reorientations	1 every 50 minutes	1 every 57.2minutes**
*n= 5 excursions in 4 cells, **n= 4 reorientations in 3 cells		

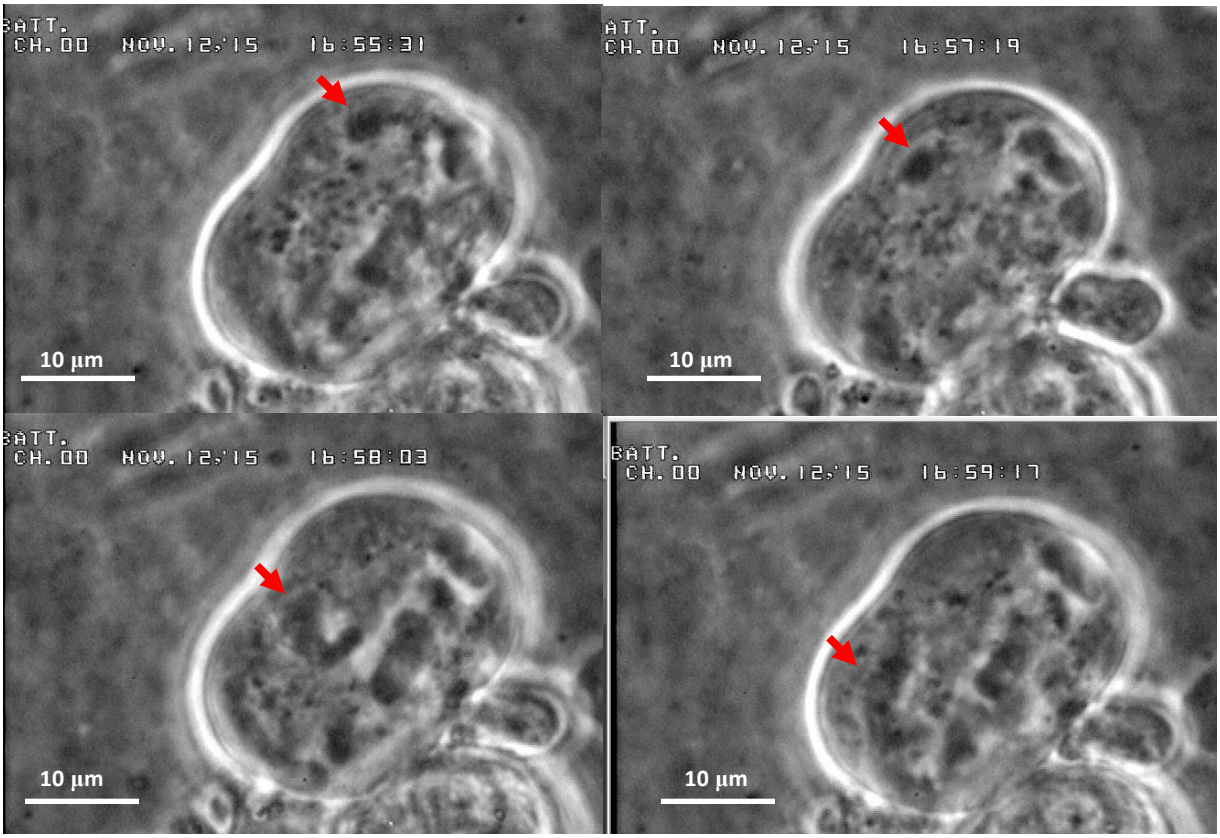


Figure 2. *Pictures taken from a video sequence of a control cell. A univalent moves from the top pole to the bottom during prometaphase. The entire movement took 4 minutes to complete. The red arrow points to the univalent that moves from the top pole to the bottom pole.*

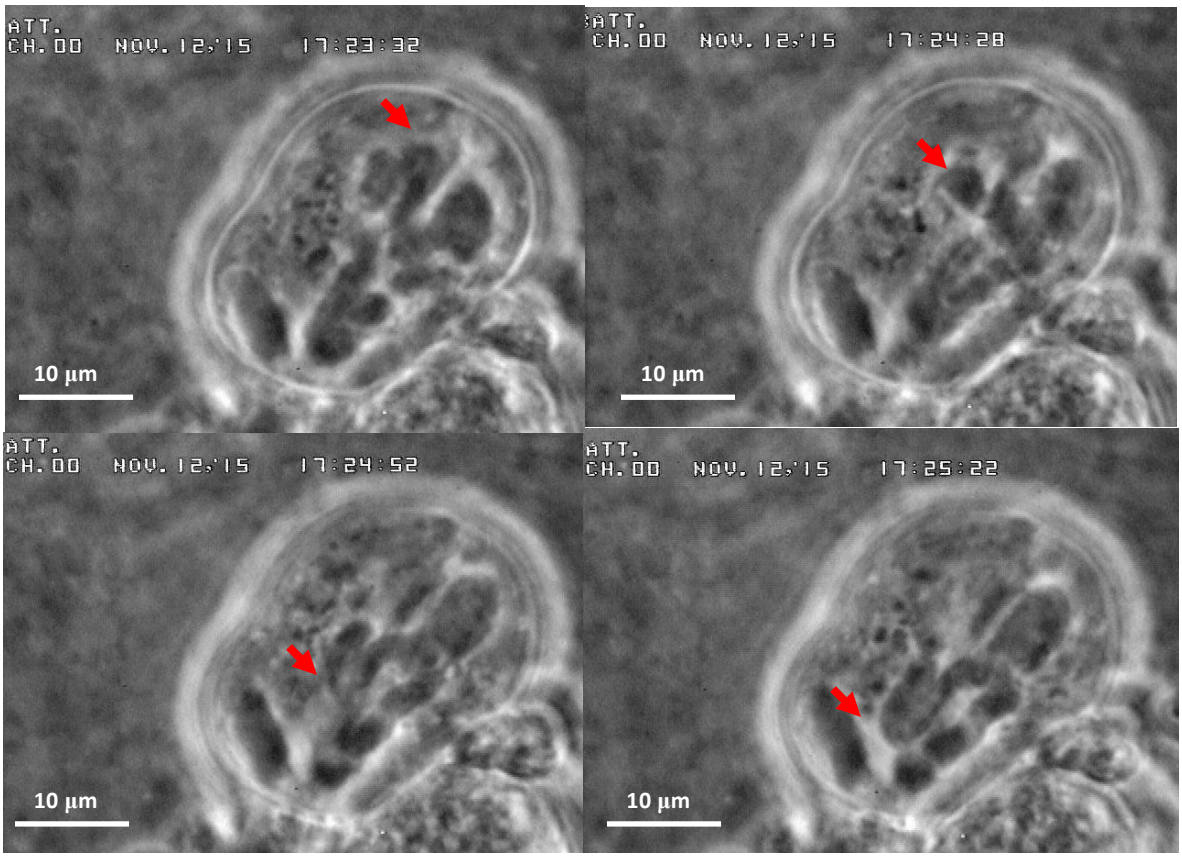


Figure 3. *Pictures from a video sequence of a control cell. The bivalent at the top starts off with both kinetochores facing a single pole. The bivalent reorients so that it is bipolar instead of unipolar. The red arrow points out the kinetochore that is originally facing one pole and then reorients to face the opposite. The movement took 3 minutes to complete.*

The goal of my experiments is to determine if altering myosin phosphorylation will affect chromosome movements. In order to do this, I need to look at changes in these parameters before and after the addition of different drugs. To see if the drug works, I first need to determine how much variation can be expected under normal cell conditions (Figure 4). The parameters all fluctuate under normal prometaphase conditions. The maximum fluctuation observed for each parameter is present in table 4.

Table 4. Summary of variations for each parameter measured in *Mesostoma ehrenbergii* primary spermatocytes during prometaphase.

Feature	**Averages observed**	**Maximum fluctuations******	**n value**
Velocity	Towards pole: 7.66 ± 2 (SD) µm/min Away: 6.44 ± 1.38 (SD) µm/min	14% ± 1.8% (SD)	30*
Amplitude	3.58 ± 0.55 (SD) µm	12% ± 3%% (SD)	30*
Period	1 minute 14 seconds	14% ± 1.6% (SD)	30*
Bivalent length	15.66 ± 1.91 (SD) µm	10% ± 2.8% (SD)	15*
Cell length	26.56 ± 1.97 (SD) µm	1% ± 0.4% (SD)	9 cells
Univalent excursions	1 every 25 minutes	N/A	4**
Bivalent reorientations	1 every 57 minutes	N/A	3***

*15 cells, 30 kinetochores ** 102 minutes of video in 4 cells, 5 excursions *** 123 minutes of video in 3 cells, 4 reorientations

**** 3 cycles measured every 10 minutes in 15 cells totalling 875 minutes

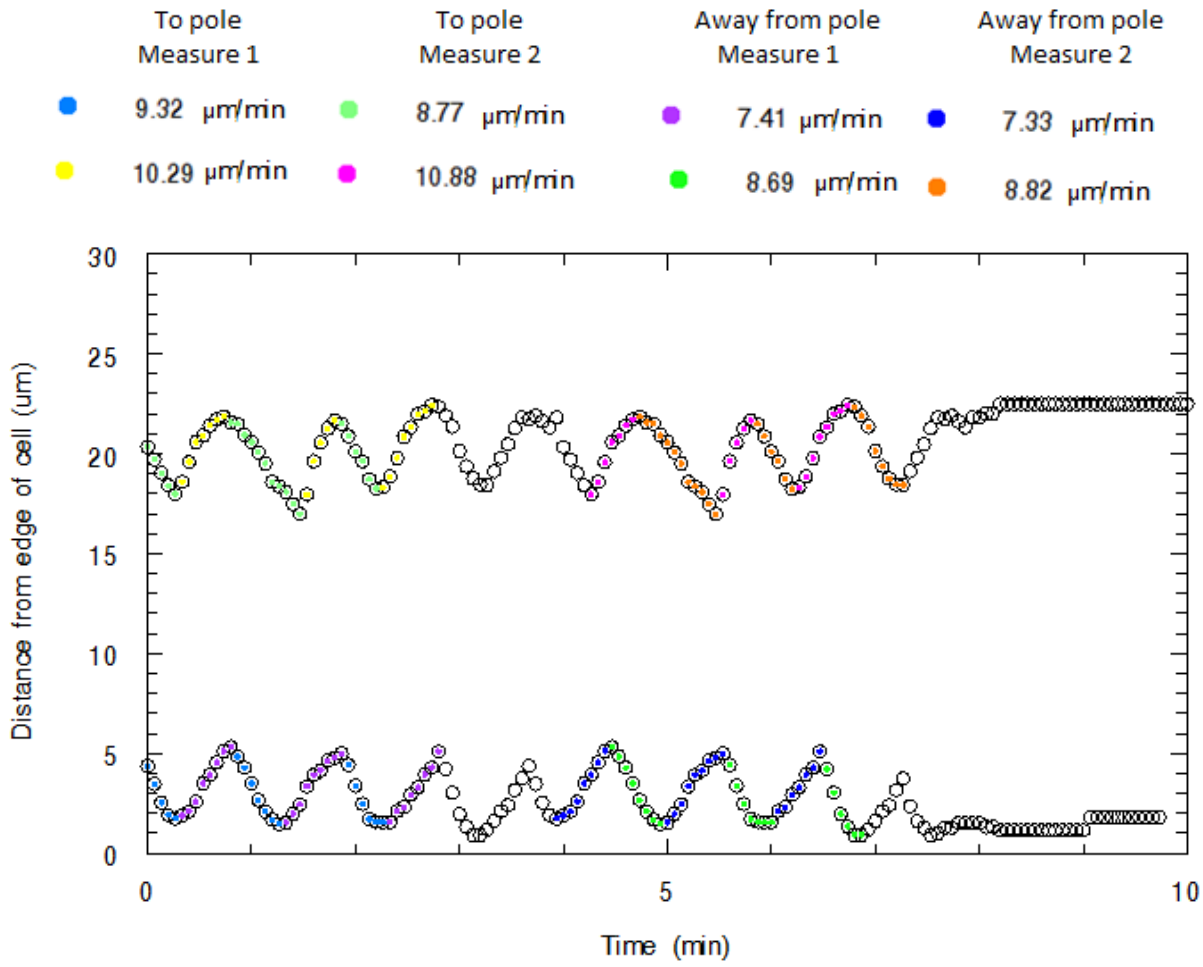


Figure 4. *Movement of a single bivalent in a primary spermatocyte control cell. The velocities of movement towards the pole as well as away from the pole were measured at two different time intervals. The cell completed anaphase at approximately 8 minutes.*

Drug Treatments

To determine the effects of different drugs on chromosome oscillations during prometaphase, I treated *Mesostoma ehrenbergii* primary spermatocytes during normal prometaphase movement. The effects that were observed after treating cells with phosphorylation inhibitors ML-7 and Y-27632 are decreases in the velocities of movement towards and away from the pole and decrease in amplitudes In cells that were treated with a calyculin A, the movement towards and away from the pole increased and the amplitude increased. All bivalents within a cell were not affected to the same degree. Sister kinetochore also were not affected to the same degree. In the presence of drugs, bivalent oscillations also became irregular and movement was much more jerky and sporadic. The period was affected only in cases where total myosin activity was perturbed. Targeting single phosphorylation pathways had no effects on bivalent oscillation periods. Bivalents were also observed to move towards a single pole while being treated by different drugs. In addition, certain drug conditions caused the number of univalent excursions and bivalents reorientations to become more or less frequent.

I first treated cells during prometaphase with different kinase inhibitors, and then, when there was a decrease in velocity and amplitude, washed out the drug to get recovery of the parameters. I wanted to determine whether these parameters were controlled by a single phosphorylation pathway or if there were additional pathways. If using multiple kinase inhibitors at once does not completely stop bivalent oscillations then there are additional pathways to phosphorylate spindle myosin. However, if the use

of multiple phosphorylation inhibiting drugs does stop chromosome oscillations then there are no additional pathways contributing to the movement.

The effects of phosphorylation pathways on univalent excursions and bivalent reorientations were observed. Primary spermatocytes were treated kinase inhibitors during prometaphase and the number of univalent movements and bivalent reorientations determined. If myosin phosphorylation is involved in these movements then a decrease or increase should be observed when phosphorylation pathways are targeted. If multiple pathways are involved in univalent excursions and bivalent reorientations then these movements should be increasingly inhibited as more phosphorylation pathways are inhibited. It may be the case that only single phosphorylation pathways are involved. In this case, the occurrence of these movements should only decrease when this phosphorylation pathway is targeted or total myosin is inhibited.

Effects of ML-7 and Y-27632 on Bivalent Oscillations in Primary Spermatocytes

In order to determine which phosphorylation pathways of myosin are involved in bivalent oscillations, I treated primary spermatocytes with ML-7 and Y-27632. ML-7 is a reversible inhibitor that targets myosin light chain kinase. It does this by preventing ATP binding through competitive inhibition (Bain et al., 2003). Y-27632 is an additional myosin inhibitor but acts through decreasing the activity of Rho-kinase (Uehata et al., 1997). I first treated primary spermatocytes with the drugs separately in order to determine if the pathways are involved in chromosome oscillations.

After I treated cells with 3 different concentrations of ML-7 (10, 15 & 20 μM) there was a noticeable decrease in chromosome velocities towards and away from the pole and in the distance they moved. Similar results were obtained when primary spermatocytes were treated with 200 μM Y-27632 (table 5). When cells were treated with only ML-7 the movement became very irregular. Kinetochores would quickly jerk back and forth during oscillations making the peaks very sharp (figure 5). Primary spermatocytes that were treated with Y-27632 showed similar effects to cells treated with ML-7 (table 5). The oscillations of the bivalents would become irregular and the bivalent would jerk back and forth instead of smooth movements. In many cases, the kinetochore would shift back and forth while either moving towards or away from the pole (figure 6).

Table 5. Effects of ML-7 and Y-27632 on bivalent oscillations in Mesostoma primary spermatocytes.

	Mesostoma Primary Spermatocyte		
	ML-7	**Y-27632**	**ML-7 & Y-27632**
Average Velocity Change	*To pole:* - 41% ± 1.4 (SE)** (range: 15-61%) *Away from pole*: -41% ± 1.3 (SE)** (range: 16-64%)	*To pole:* -38% ± 1.8% (SE)** (range: 26- 61%) *Away from pole*: -36% ± 1.9% (SE)** (range: 16- 64%)	*To pole:* -39% ± 1% (SE)** (range: 22- 52%) *Away from pole:* -40% ± 0.6% (SE)** (range: 26-53%).
Average Amplitude Change	-46% ± 1.4% (SE)** (range: 21-66%)	-40% ± 1.8% (SE)** (range: 24- 73%)	-41% ± 2.3% (SE)** (range: 16-71%).
Number of cells	20	11	6
*Number of Kinetochores**	32	23	12
*represents n value, ** $p \leq 0.05$ 3 cycles measured while drug present (approximately 5 minutes)			

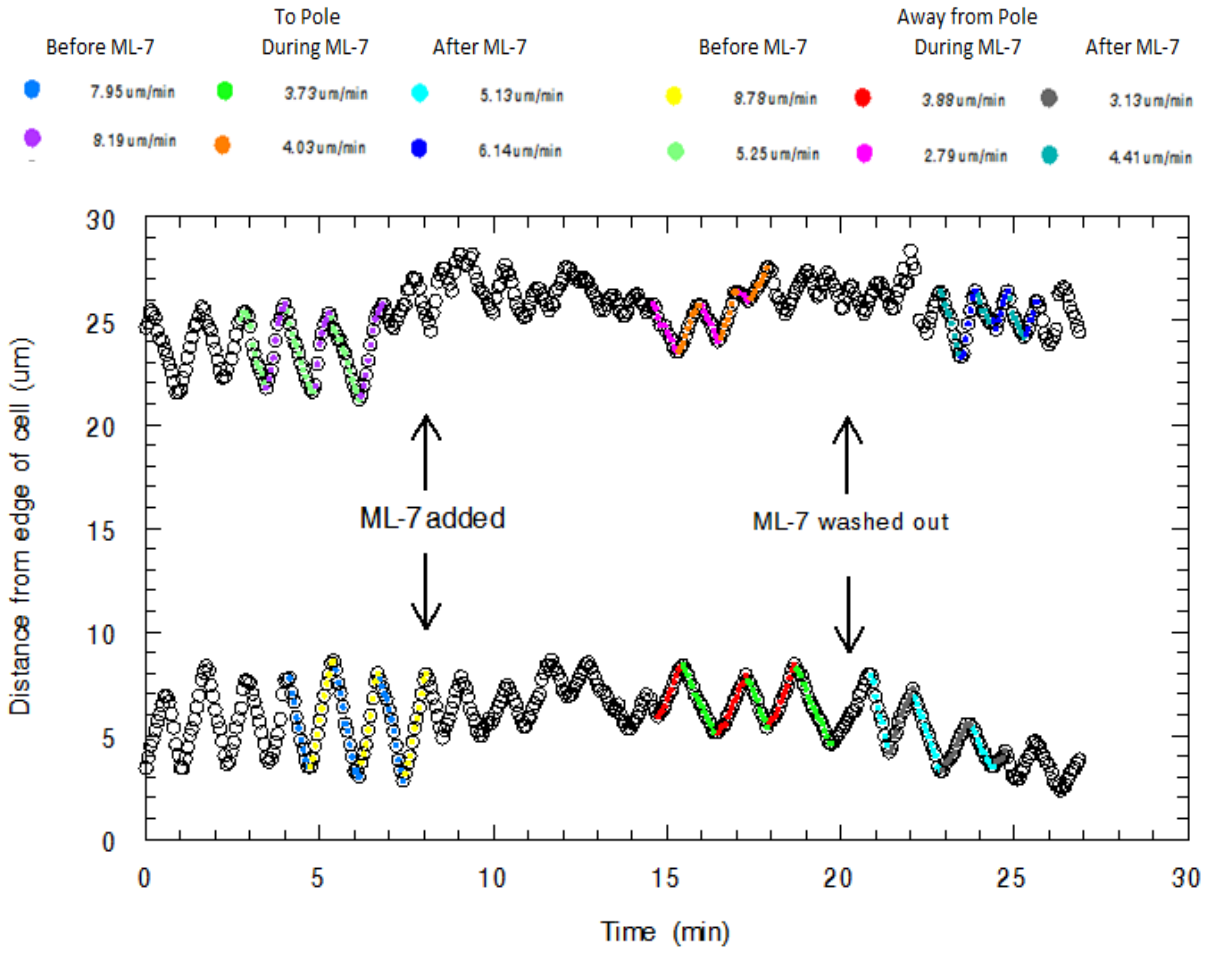


Figure 5. *Movement of a single bivalent in a primary spermatocyte. The cell was treated with 20 μM ML-7 for 12 minutes before wash out. With ML-7 present the movement of the kinetochores become irregular and slowed. The amplitudes of the oscillations also decrease and are not consistent while ML-7 is present. After wash out the movement becomes more regular with an increase in both the velocity and amplitude.*

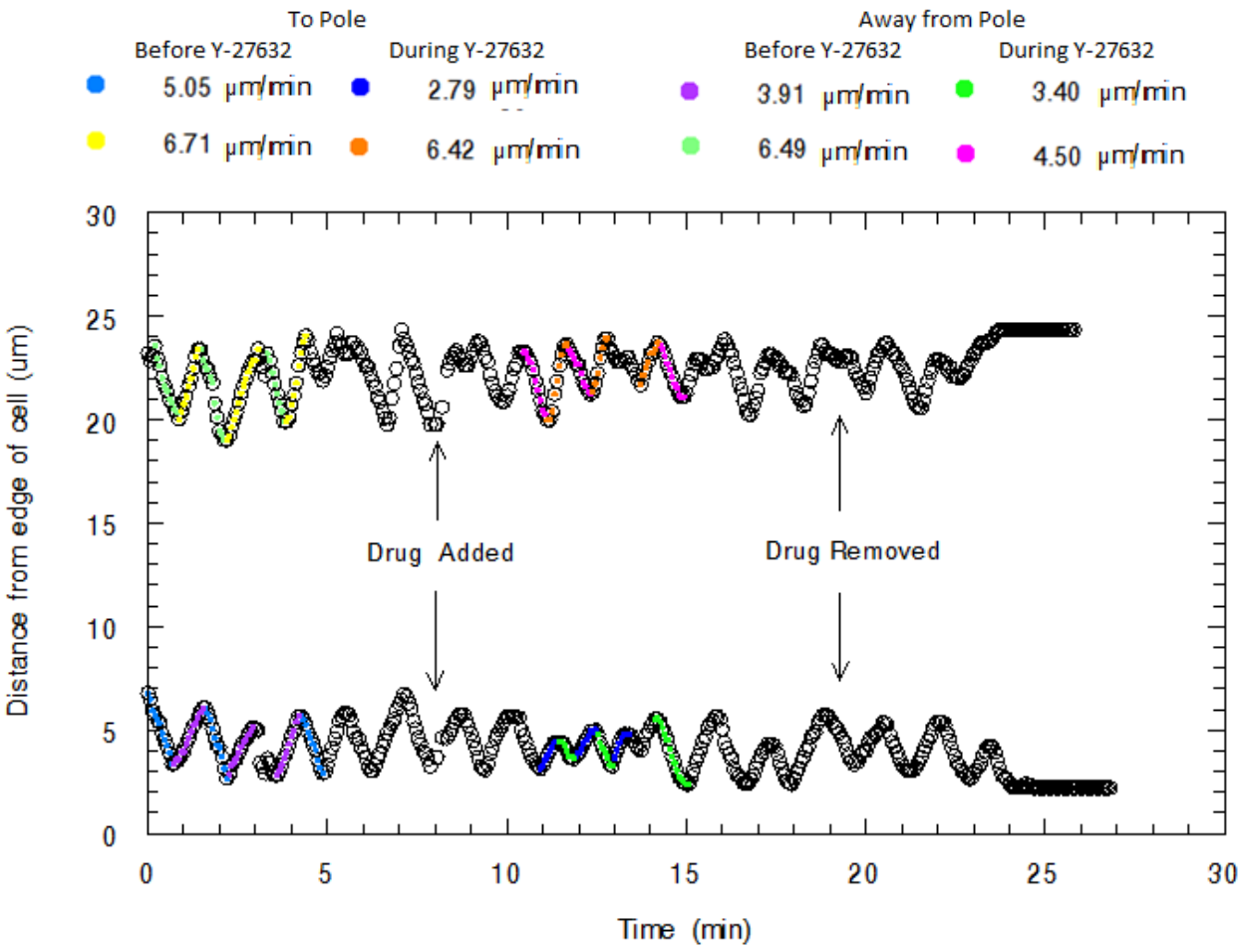
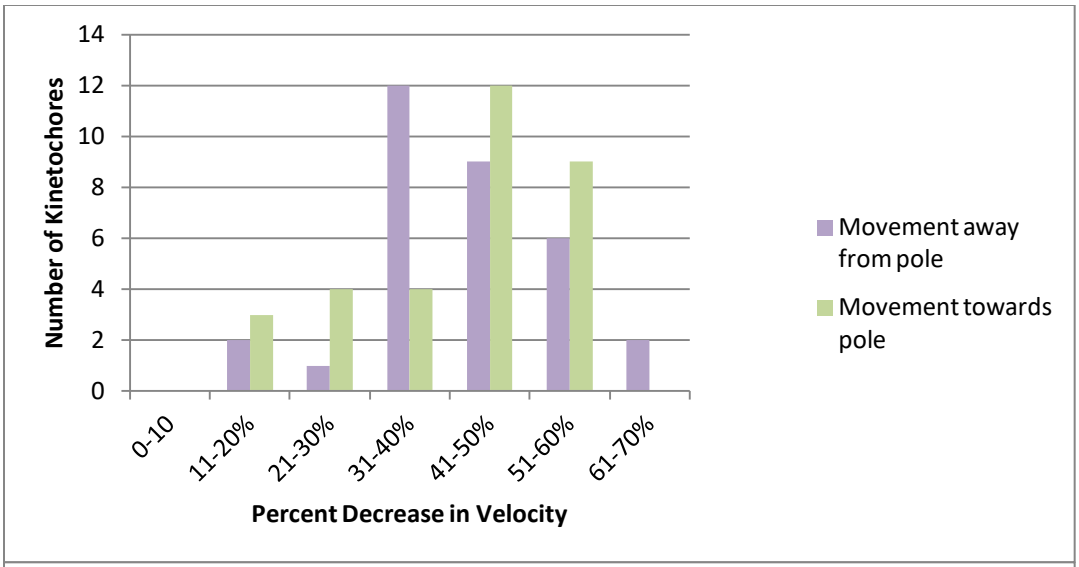


Figure 6. *The graph shows the movement of a single bivalent in a primary spermatocyte treated with 200* µ*M Y-27632. A minute after Y-27632 was added to the cell the movements of the kinetochores became irregular and slowed down. After 2 minutes of Y-27632 being added the amplitudes of the oscillation decreased. After the cell was treated for 11 minutes, Y-27632 was washed out and 3 minutes later the cell proceeded into anaphase.*

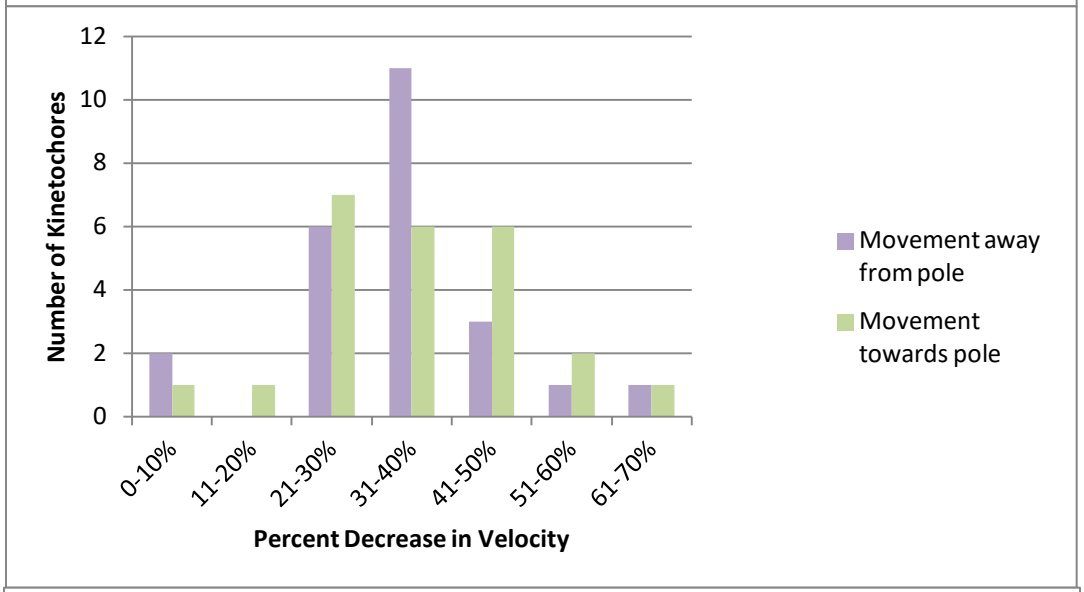
ML-7 affected one direction of movement slightly more than the other. The majority of the kinetochores velocities towards the pole decreased by 41-50%, while most of the movement away from the pole decreased by 31-40% (figure 7-A). Y-27632 seemed to have the opposite effect as the movement away from the pole was perturbed more compared to the movement towards the pole (figure 7-B).

When ML-7 and Y-27632 were washed out from cells recovery was observed within 1-2 minutes. After the primary spermatocytes were exposed to ML-7 for longer than 15 minutes, 8 out of 28 cells died. The furrow wwas completely lost, and bivalents stopped moving or curled up. This did not happen when cells were treated with Y-27632 for longer periods of time, or when both of the drugs were used together. When Y-27632 was washed out from the cell there was better recovery with many of the parameters returning to almost 100% of the original value.

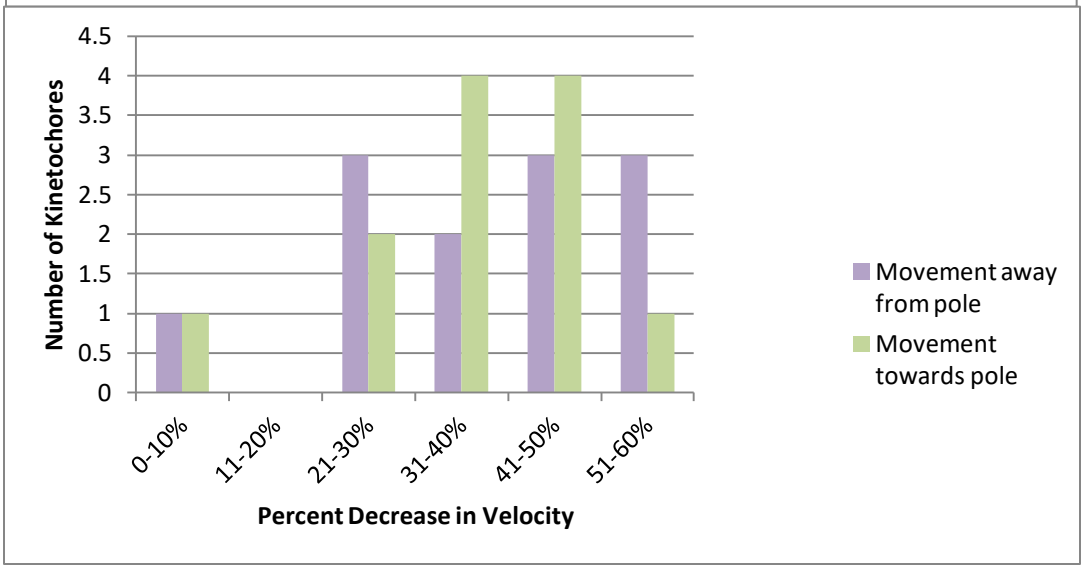
A


Number of Kinetochores
14
12
10
8
6
4
2
0
0-10
11-20%
21-30%
31-40%
41-50%
51-60%
61-70%
Percent Decrease in Velocity
Movement away from pole
Movement towards pole

B


Number of Kinetochores
12
10
8
6
4
2
0
0-10%
11-20%
21-30%
31-40%
41-50%
51-60%
61-70%
Percent Decrease in Velocity
Movement away from pole
Movement towards pole

C


Number of Kinetochores
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4
3.5
3
2.5
2
1.5
1
0.5
0
0-10%
11-20%
21-30%
31-40%
41-50%
51-60%
Percent Decrease in Velocity
Movement away from pole
Movement towards pole

Figure 7.A: *Distribution of percent decrease in velocity of kinetochores treated with ML-7.* ***B:*** *Distribution of percent decrease in velocity of kinetochores treated with Y-27632.* ***C:*** *Distribution of percent decrease in velocity of kinetochores treated with 200* µM *Y-27632 and 20* µM *ML-7.*

The bivalents in cells that were treated with ML-7 would also shift towards a single pole (figure 8). This would be observed within 2 minutes of the drug being added and the bivalent would remain in that position while the drug was present. Once ML-7 was removed the bivalents would move towards their original positions or would shift even farther away. The same trend was observed in cells treated with only Y-27632. The main difference from ML-7 is that when Y-27632 was washed out the bivalent would move back to the original position.

Furrow regression was also observed in cells that were treated with ML-7 (figure 9). In the highest concentration used (20 µM), the furrow would be completely lost and the cell would become very rounded many times not being able to recover. At lower concentrations, the cell would turn into an oval shape and would recover when ML-7 was washed out. Furrow regression in cells treated with Y-27632 was not as drastic compared to ML-7 and in many cases was one sided (figure 10).

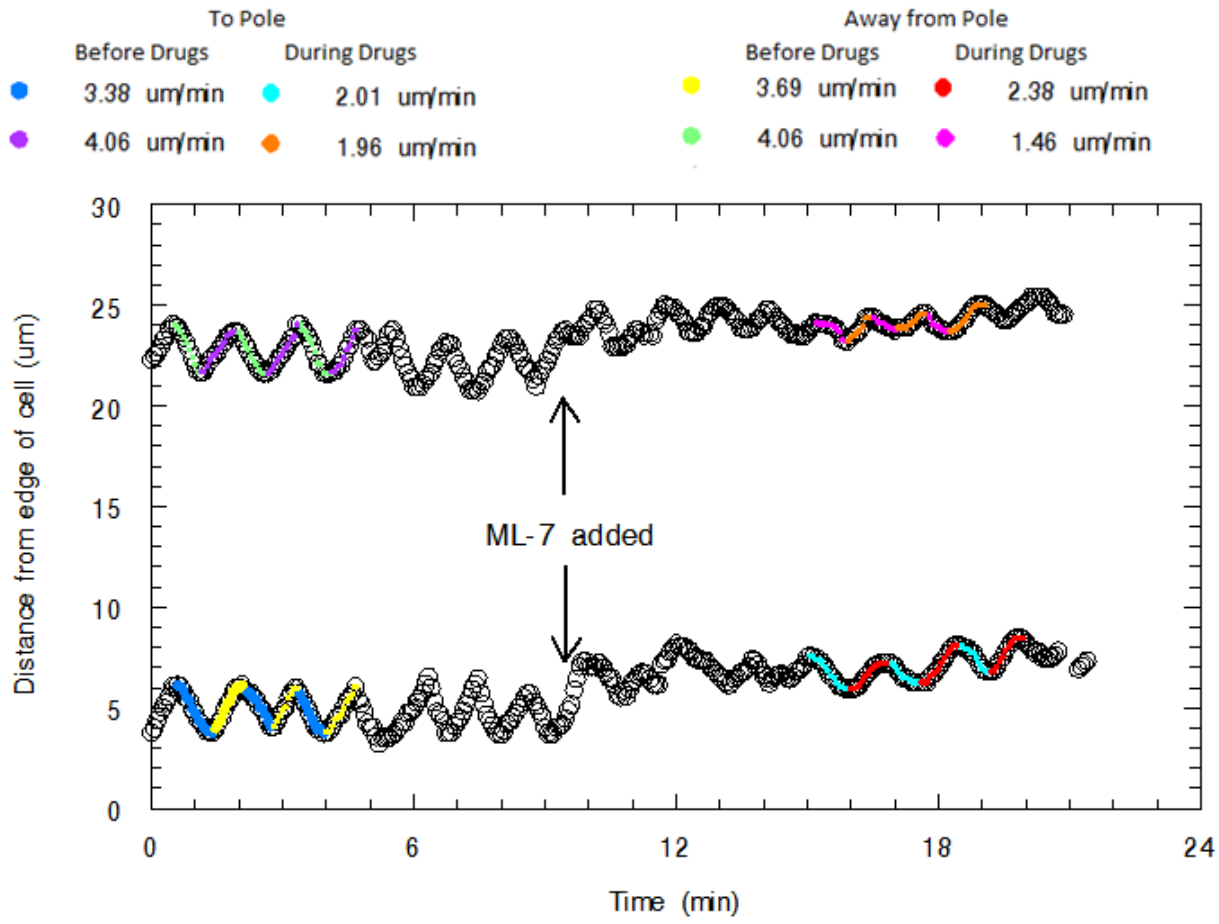

Figure 8. *The graph depicts a single bivalent in a cell that was treated with 10 μM ML-7. When ML-7 was added to the cell the velocity and amplitude of the kinetochores decreased. The bivalent oscillations shifted towards the top pole about a minute after ML- was added and continued to oscillate there for the remainder of the time.*

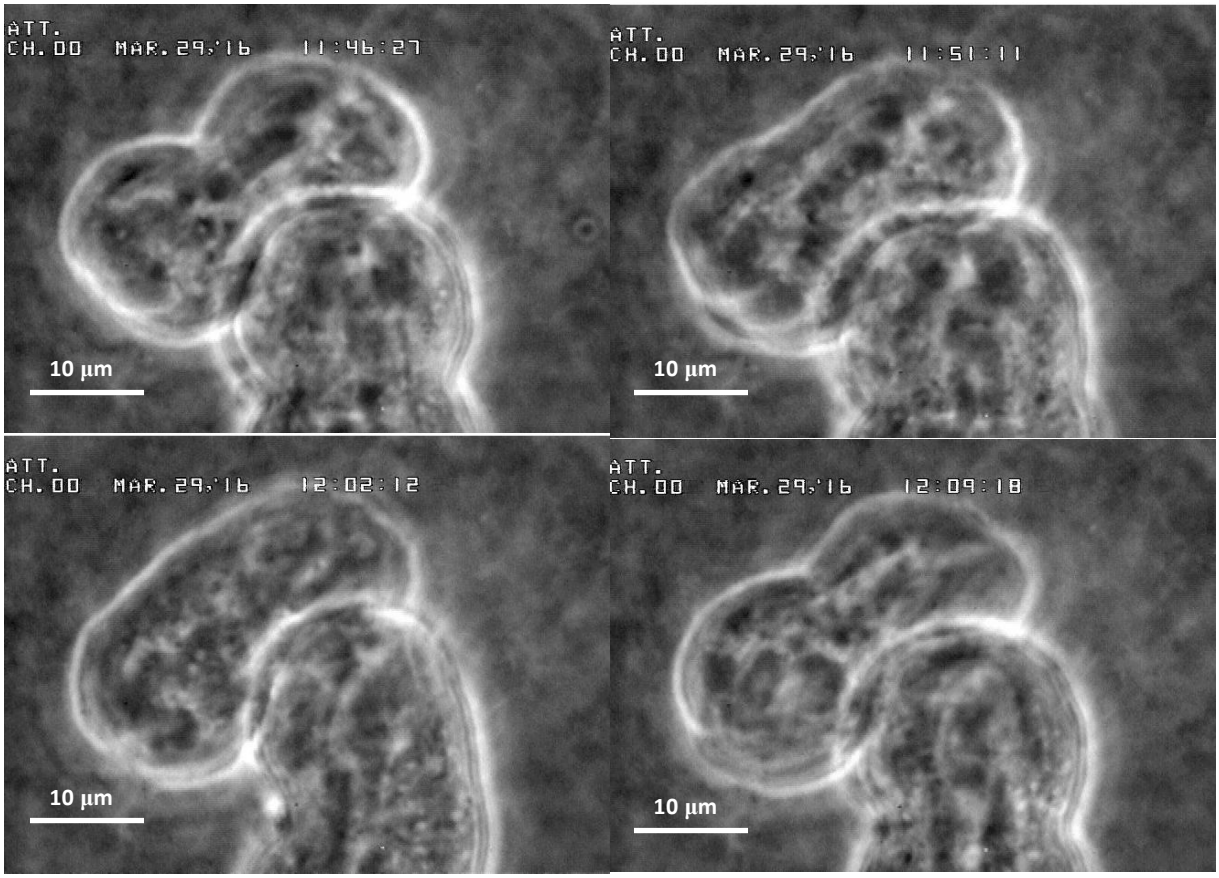

Figure 9. *Pictures from a video sequence of furrow regressing with the addition the 10 µM ML-7. The cell is treated with ML-7 at 11:47:57 and the furrow begins to regress in less than 2 minutes. Once the drug is washed out at 12:02:15 the furrow begins to ingress back to normal width.*

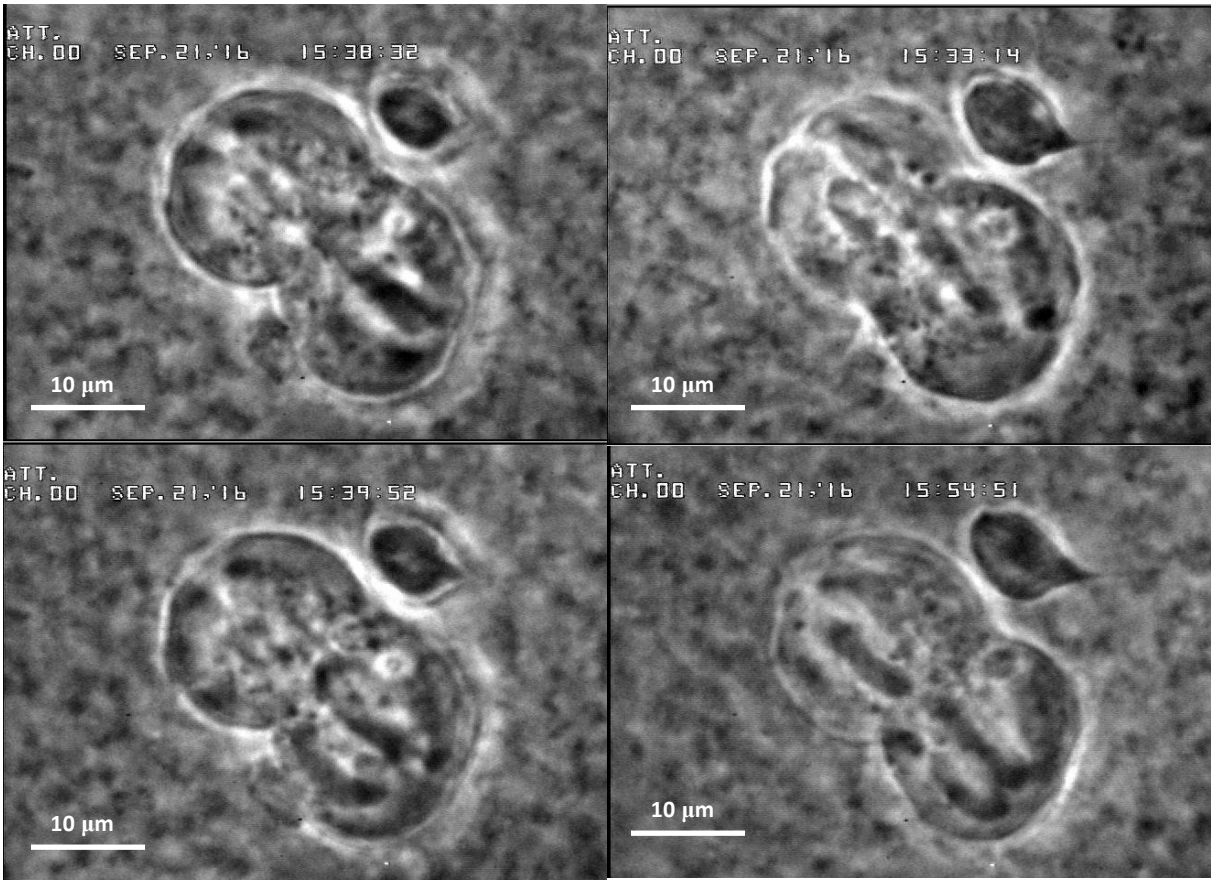

Figure 10. *Pictures from a video sequence of a cell treated with 200 µM Y-27632 added at 15:37:40. The furrow begins to regress about 1 minute after Y-27632 is added. The furrow regresses more on the right side of the cell compared to the left. Y-27632 is washed out at 15:53:39 the furrow begins to ingress back to normal width.*

Bivalent lengths were not altered by the presence of either ML-7 or Y-27632, but the length of the cell was. Shortly after the drugs were added (1-2 minutes), the cell would decrease in length (table 6).

Table 6. Effects of ML-7 and Y-27632 on the length of the primary spermatocytes in *Mesostoma ehrenbergii.*

Treatment	ML-7	Y-27632	ML-7 & Y-27632
Percent decrease in cell length	4% ± 0.04 (SE)** (range: 2- 6%)	5% ± 0.03% (SE)*** (range: 2- 8%)	5% ± 0.01% (SE)** (range: 4-7%)
Number of cells with change*	18	11	6
Number of cells without change	2	0	0
*represents n value, ** $p \leq 0.01$, *** $p \leq 0.05$			

After Y-27632 was washed 7 out of 10 cells would go into anaphase within 15 minutes of the drug being washed out. In all 7 cells, there was never a univalent excursion or bivalent reorientation (figure 11). In the 3 cells that did not proceed to anaphase once Y-27632 was removed, both univalent excursions and/or bivalent reorientations occurred. If one of these phenomena occurred while Y-27632 was present in the cell then anaphase would not occur (table 7). One cell was followed for an hour and a half after Y-27632 had been washed out and anaphase had still not occurred. A total of 558 minutes of video was recorded for cells treated with Y-27632

(includes before, during and after drug treatment). In this time only 4 univalent excursions occurred and 2 bivalent reorientations, all of which occurred in 3 cells. This corresponds to a univalent excursion every 149.5 minutes and a bivalent reorientation every 279 minutes.

Table 7. Effect on univalent movements in the presence of 200 µM Y-27632.

Treatment	Anaphase	Number of cells	Univalent movement	Bivalent reorientation
200 µM Y-27632 (n= 11 cells)	Occurs within 15 minutes of drug removal	7	0	0
	Does not occur within 15 minutes of drug removal	3	4	2

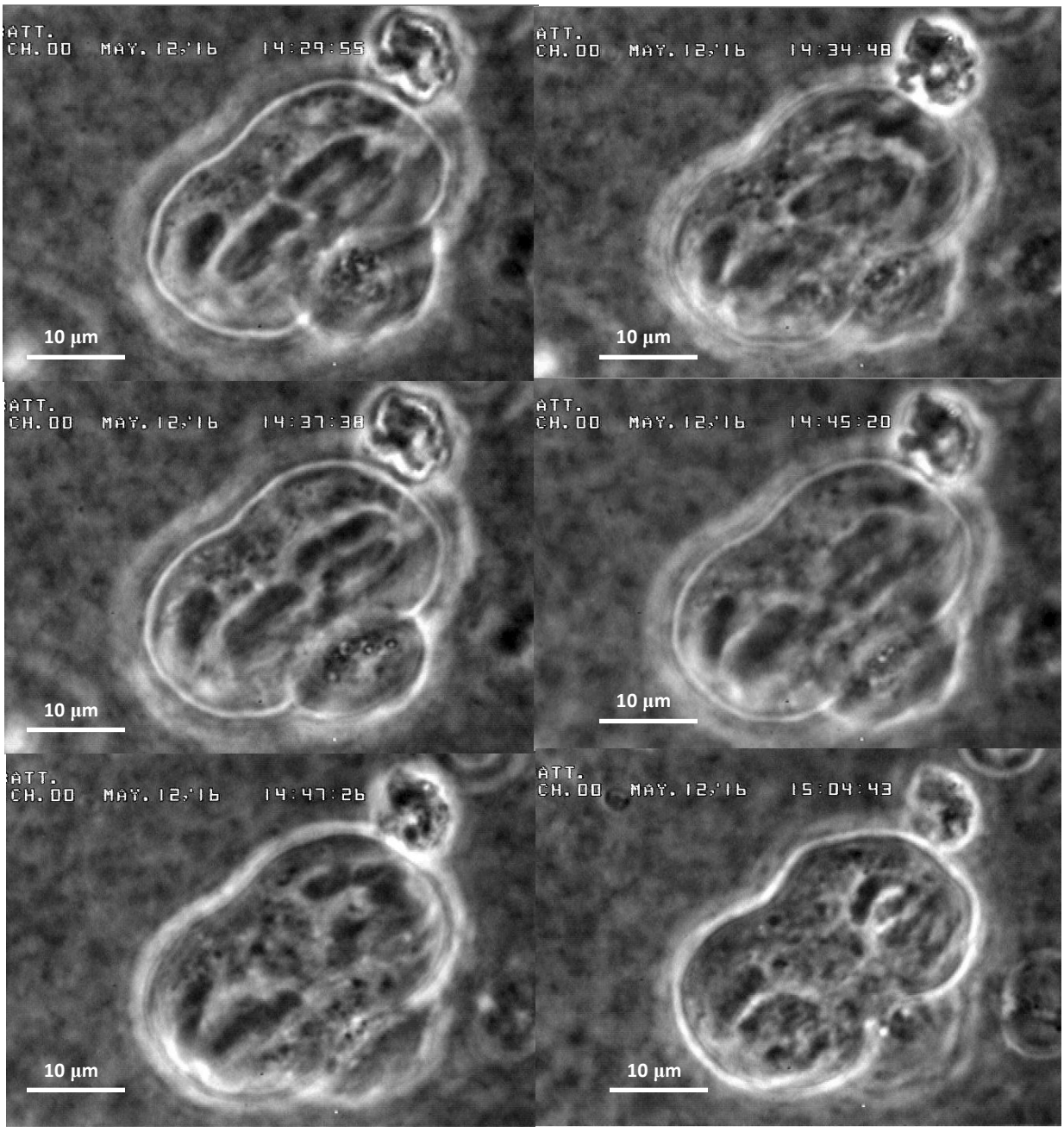

Figure 11. *Pictures from a video sequence of a cell treated with 200* µ*M Y-27632 at 14:36:50. No univalent movements are observed while the cell is treated with Y-27632. At 14:44:10, Y-27632 is washed out from the cell and within a minute anaphase occurs.*

I then wanted to test whether inhibiting both of these pathways would cause chromosome oscillations to completely stop. The movements, however, did not completely stop suggesting additional phosphorylation pathways. The velocity and amplitude also decreased by approximately the same amount when cells were treated with ML-7 and Y-27632 separately which was not expected (table 5). Since both ML-7 and Y-27632 added separately had a significant effect, adding both inhibitors together was expected to have almost a doubling effect. When both ML-7 and Y-27632 were added together bivalent oscillations were not as irregular. The movements were smoother and the kinetochore would not shift back and forth while either moving to or from the pole (figure 12). In cells that were treated with both kinase inhibitors, one sister kinetochore was usually affected more than the other. In most cases, the movement of one kinetochore would almost completely stop and it would just vibrate minimally back and forth.

When primary spermatocytes were treated with both kinase inhibitors movements both towards and away from the pole were equally affected (figure 7-C). This was expected since ML-7 had a larger effect on the movement towards the pole and Y-27632 effected the movement away from the pole more greatly.

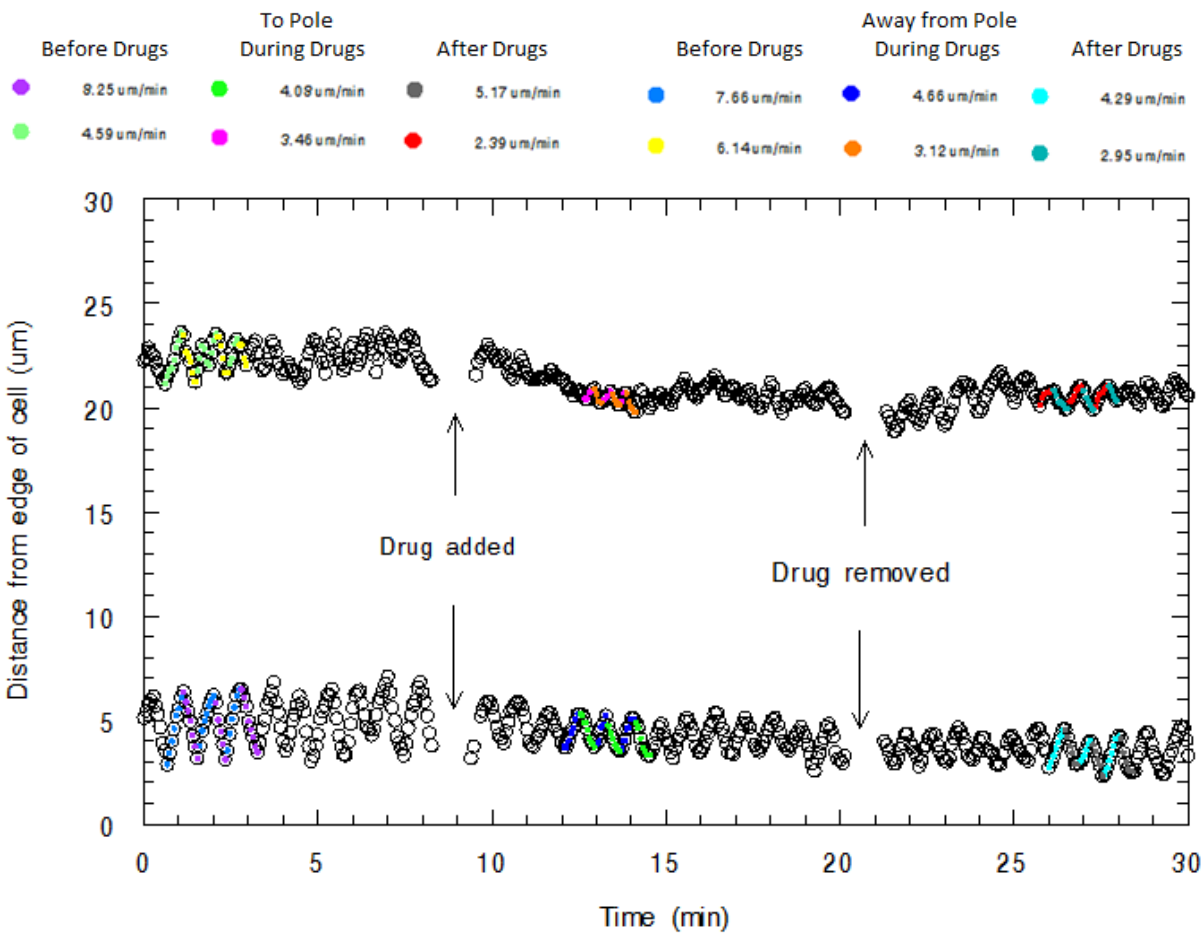

Figure 12. *Movement of a single bivalent in a primary spermatocyte treated with 20 μM ML-7and 200 μM Y-27632 for a minimum for 11 minutes before being washed out. Both the velocity and the amplitude decreased 2 minutes after ML-7 and Y-27632 were added. No change in the period was observed.*

With both kinase inhibitors present, both sides of the furrow were affected in a similar manner (figure 13). These results are similar to when the cell was treated with ML-7. Shortly after the addition of Y-27632 and ML-7, the furrows rapidly regressed within 2 minutes. This is in contrast to when cells were treated with Y-27632 alone and only one side of the furrow regressed. These results suggest that the drugs are affecting myosin phosphorylation. In addition to furrow regression, the length of the cell decreased similar to when ML-7 and Y-27632 were added separately (table 6).

In 6 cells that were treated with both ML-7 and Y-27632, 10 univalent excursions occurred while ML-7 and Y-27632 were present. This corresponded to a univalent movement every 18.1 minutes of video. Similar to when the cells were treated with Y-27632 alone, anaphase was not observed after washout, except in two cells. Both of these cells had an even number of univalent excursions, where a univalent would travel to the opposite pole and then the same univalent would travel back (figure 14). In one cell this sequence occurred once while in the other the sequence occurred twice with the same univalent. Once ML-7 and Y-27632 were washed out, both of these cells proceeded into anaphase within 50 minutes. Bivalent reorientations were also observed in 2 of the cells treated. This corresponds to a bivalent reorientation once every 51.5 minutes of video. Both of these cells did not proceed into anaphase after wash out.

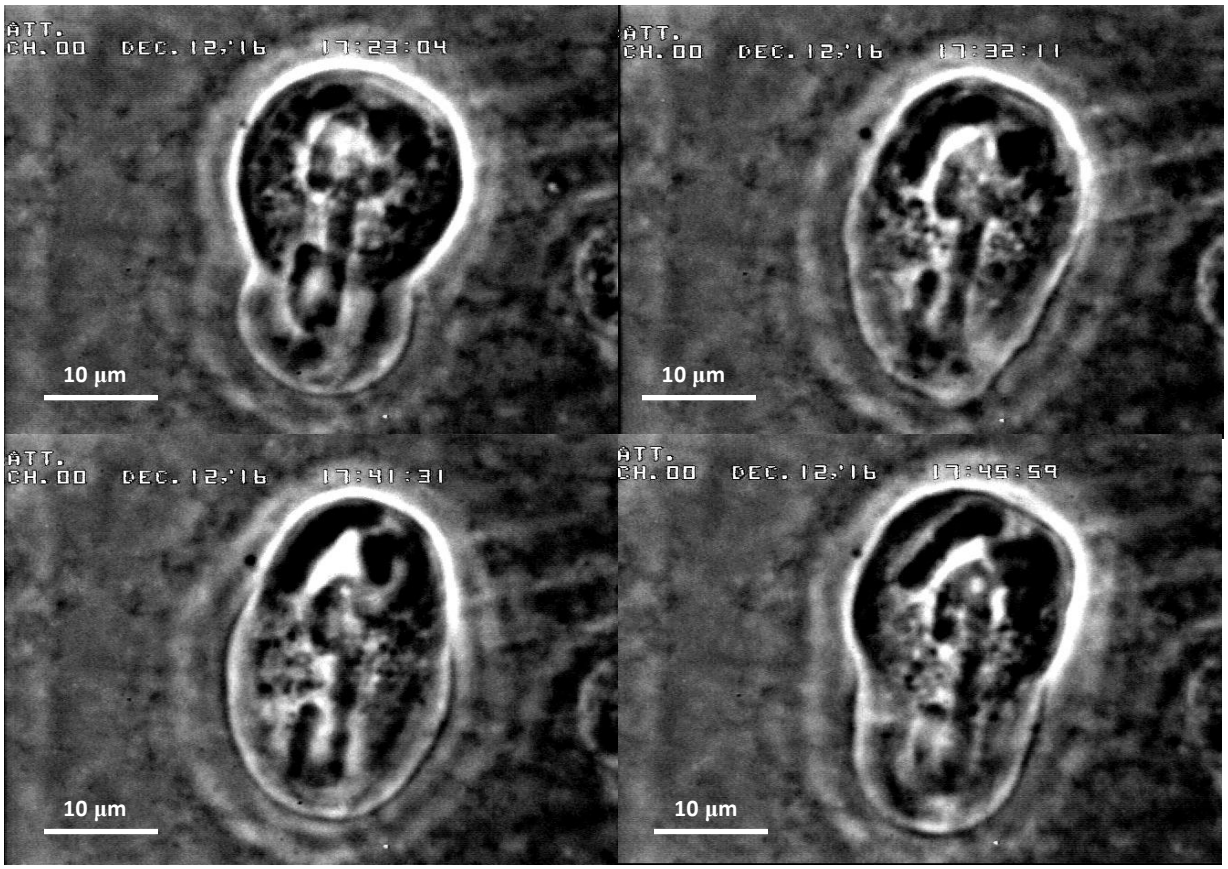

Figure 13. *Pictures from a video sequence of a primary spermatocyte treated with 20* µ*M ML-7 and 200* µ*M Y-27632. Both 20* µM *ML-7 and 200* µM *Y-27632 are added at 17:27:44 and furrow regression is observed within 1 minute. Once ML-7 and Y-27632 are removed 17:39:53 the furrow begins to ingress and recover.*

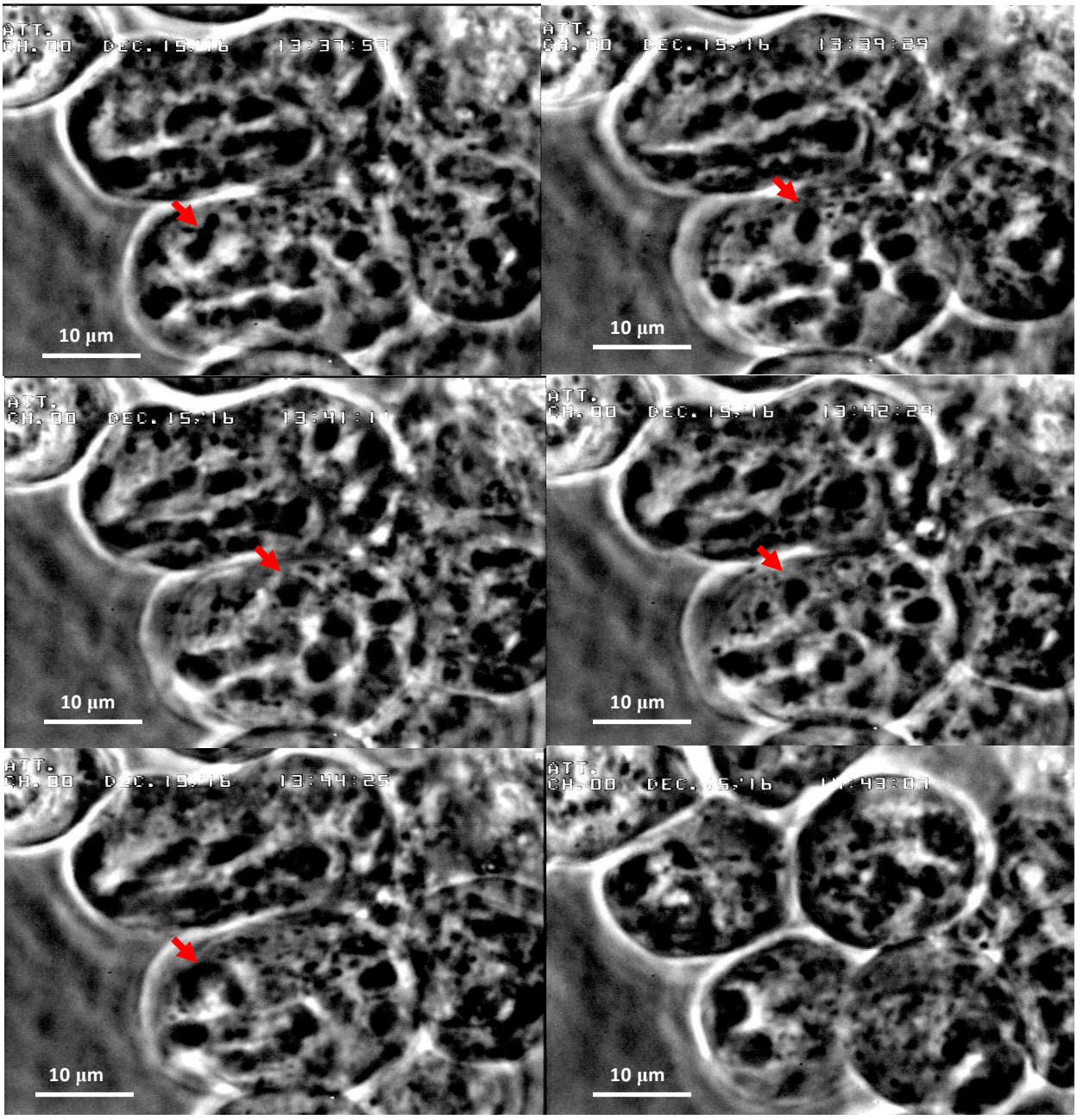

Figure 14. *While Y-27632 is present in the cell univalents excursions may or may not occur. The bottom cell has an even number of univalent movements before the drug is washed out, and completes anaphase after the drug is washed out13:48:49. The red arrow is indicating the location of a single univalent in the cell.*

H-7 as a Control for ML-7 Effects on Bivalent Oscillations

ML-7 is a potent kinase inhibitor and can also affect the activity of PKA and PKC (Saitoh et al., 1987; Quick et al., 1992; Silverman-Gavrila & Forer, 2001). In order to determine if my results were a result of myosin light chain kinase inhibition, I used H-7. H-7 also affects the same kinase but has a much higher affinity for PKA and PKC. H-7 had no major effect on bivalent oscillations in primary spermatocytes (figure 15, table 8). Therefore the effects observed in chromosome oscillations are as a result of myosin light chain kinase inhibition and not PKA or PKC. H-7 had no observable effects on bivalent length, cell length, or furrow movement. The occurrence of univalent movements and bivalent reorientations was not affected by the presence of H-7.

Table 8. Effect of 20 µM H-7 on bivalent oscillations.

Treatment	Parameter	Number of cells	Number of Kinetochores			
			Total	Increase	Decrease	No change
20 µM H-7	Velocity	6	13	1	0	12
	Amplitude			0	1	12
	Period			0	1	12

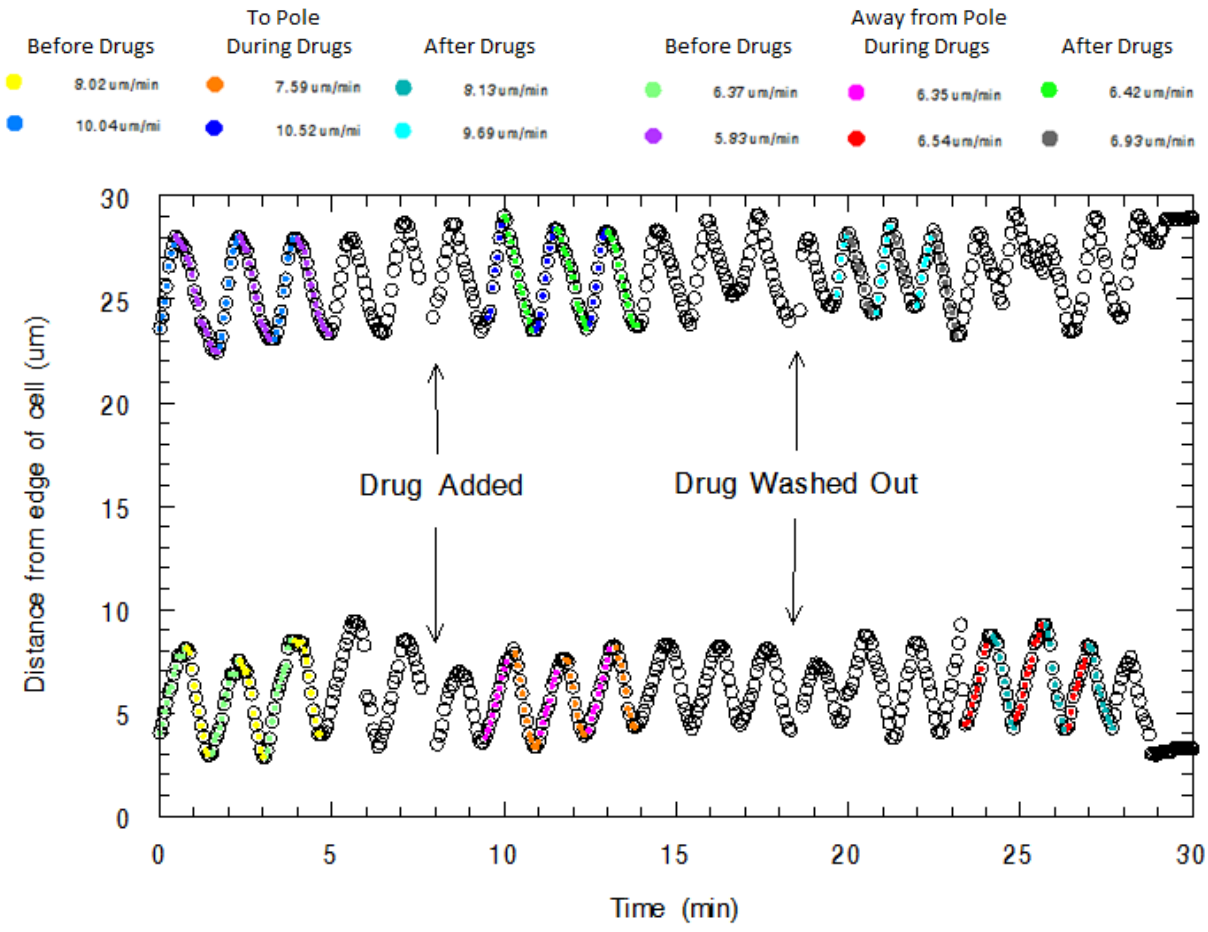

Figure 15. *The graph shows the movement of a single bivalent in a primary spermatocyte. The cell was treated with 20 μM H7 for 10 minutes prior to washout. No significant change was observed in the velocity, amplitude or period.*

Effects of BDM in Primary Spermatocytes during Prometaphase

BDM, an uncompetitive inhibitor, binds to myosin in a different region from the ATP binding site (Herrmann et al., 1992). BDM effects myosin activity not through inhibiting ATPase activity, but by preventing the power stroke and does this by stabilizing the ADP and inorganic phosphate complex (Forer and Fabian, 2005). When I treated *Mesostoma* primary spermatocytes with 20 mM BDM, there was a decrease in the velocity of the movement towards and away from the pole. In addition, the amplitude of the movement decreased and there were noticeable effects on the period of oscillations (table 9). The effect of the drug was observed within 1 minute of 20 mM BDM treatment. Bivalents within a single cell were not affected equally. The movement of one bivalent can almost completely stop while the others are only decreased (figure 16).

Table 9. Effect of 20 mM BDM on bivalent oscillations in prometaphase.

	20 mM BDM Treatment		
	Velocity	*Amplitude*	*Period*
Average Change	Towards pole: -48% ± 1.2% (SE)** Away from Pole: -47% ± 1.7% (SE)**	- 62% ± 2.9% (SE)**	Decreased 52% ± 0.5% (SE)*** Increased 23% ± 0.12% (SE)***
Number of Kinetochores*	19	19	Decrease: 8 Increase: 8
Range	Towards pole: 34 – 64% Away from pole: 30 – 72%	44 – 87%	Decrease: 43 – 59% Increase: 20 – 27%
*represents n value, ** $p \leq 0.01$, *** $p \leq 0.05$			

The velocities of the movement towards and away from the pole are affected in a similar manner. Total myosin inhibition did not seem to effect one direction more than the other (figure 17). For the movement away from the pole there was a higher number of kinetochore that decreased by 51-60%, but overall the distribution was fairly even.

BDM did not have the same effect on the period for all of the kinetochores treated (table 10). The period of the kinetochores would either increase or decrease when treated with 20 mM BDM. Sister kinetochores were not affected the same as the period of one would increase while the other decreased.

Table 10. Effect of 20 mM BDM on oscillation periods in prometaphase.

Treatment	Number of cells	Number of bivalents	Number of Kinetochores			
			Total	Increased	Decreased	No Change
20 mM BDM	10	10	19	8	8	3

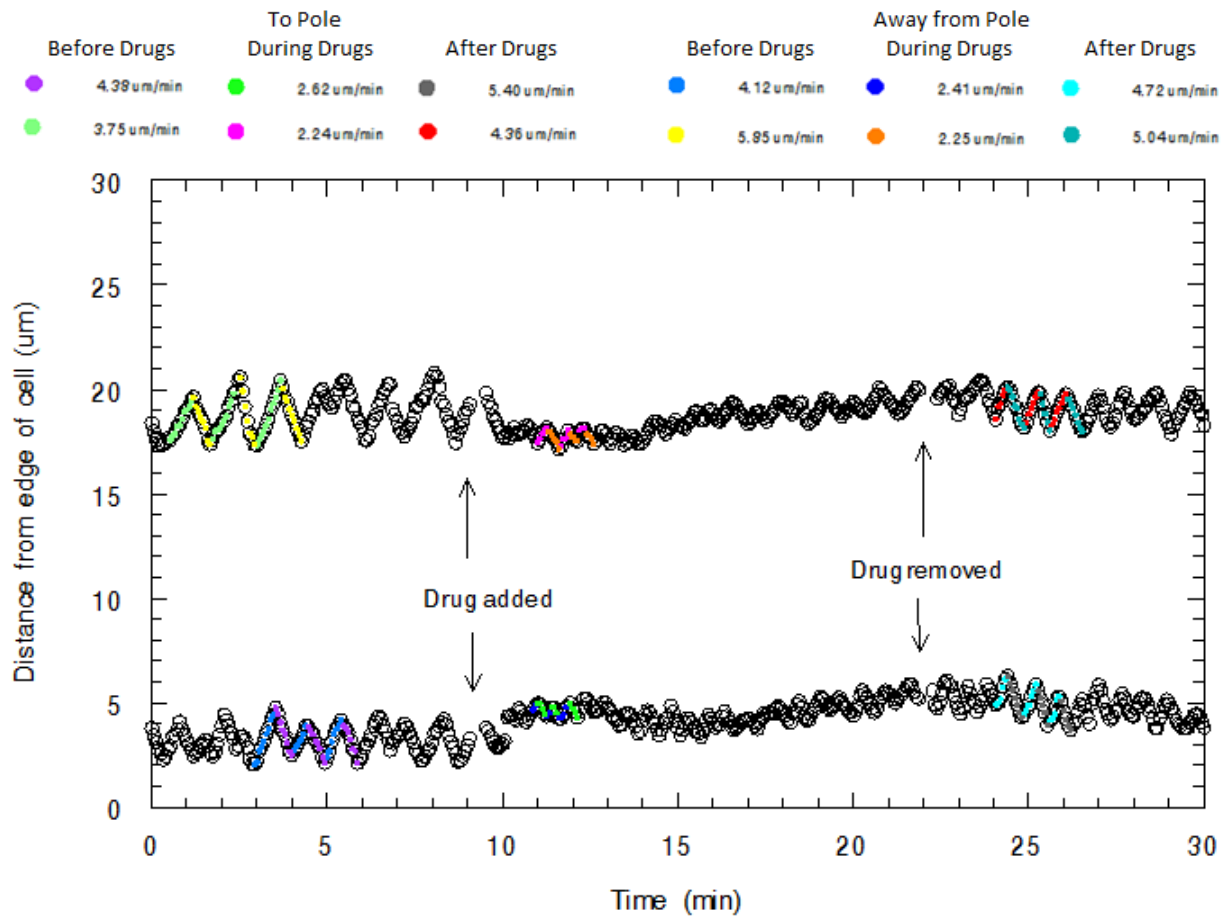

Figure 16. *Movement of a single bivalent in a primary spermatocyte treated with 20 mM BDM. The cell was treated with 20 mM BDM for 13 minutes before washout. The velocity, amplitude and period all decreased with 20 mM BDM present. After wash out, the velocity, amplitude and period recovered and movement became more regular.*

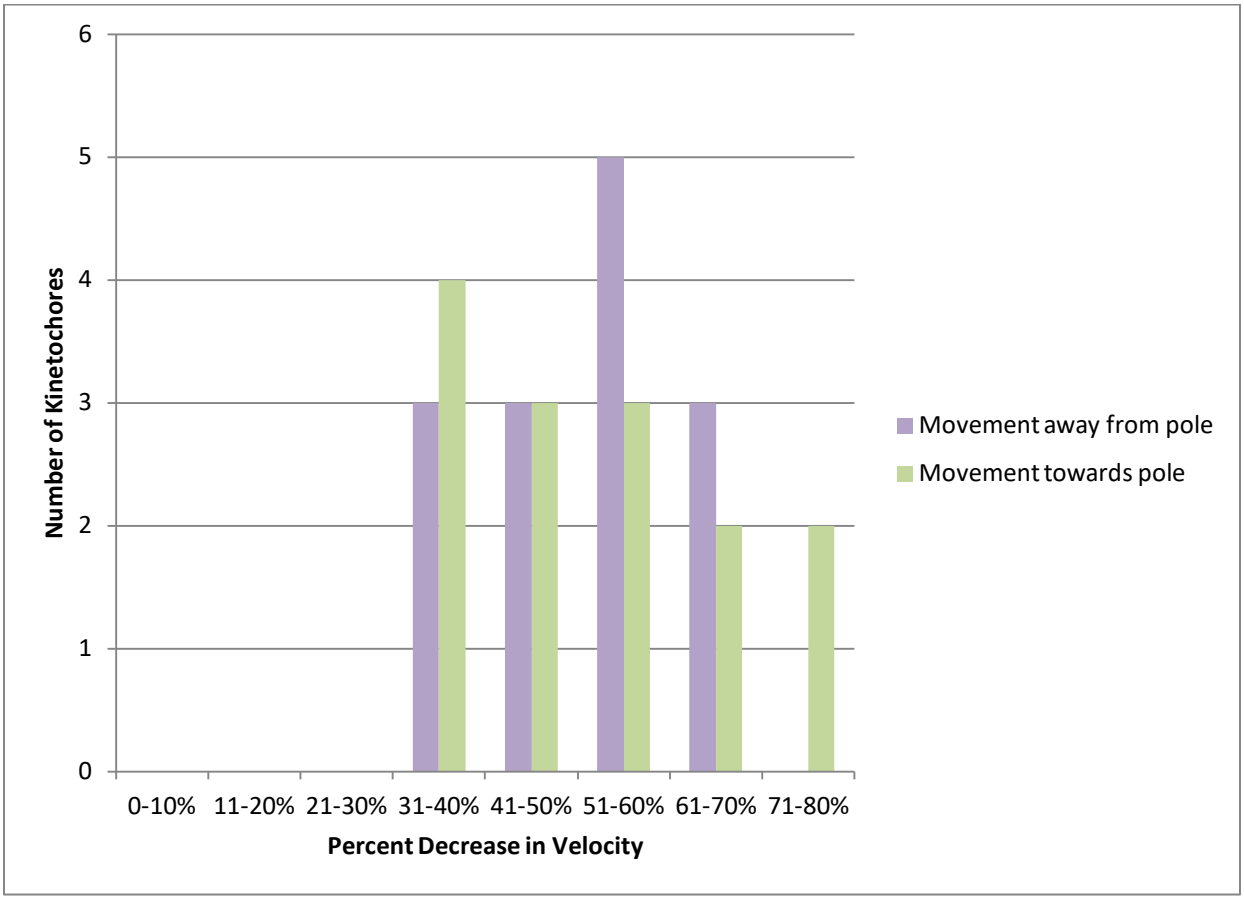

Figure 17. *Distribution of percent decrease in velocity of kinetochores treated with 20 mM BDM. The distribution is of 19 kinetochores in 10 cells.*

When BDM was washed out, the velocity, amplitude and period of the oscillations would recover within 1-2 minutes. Recovery could only be measured in 4 of the cells treated as the bivalents began to curl and the cells would begin to die after treatment periods of longer than 10 minutes (table 11).

Table 11. Recovery of bivalent oscillations after 20 mM BDM wash out.

			Number of Kinetochore		
	Number of cells	Average Recovery (compared to original value)	Total	Recovery	No Recovery
Velocity	4	Towards pole: 26% Away from pole: 92%	8	Towards pole: 6 Away from pole: 8	Towards pole: 2 Away from pole: 0
Amplitude	4	72%	8	8	0
Period	4	81%	8	4	4

The length of the bivalents and cell were also affected by 20 mM BDM treatment (table 12). The longer 20 mM BDM was present, the length of the bivalent would continue to increase, except for one bivalent where no increase in length was observed. When BDM was washed out only 1 bivalent began to decrease to the original length (figure 18) while the others maintained the new length. The length of the cells would decrease with the addition of BDM a minute after treatment but would increase to their normal size once BDM is removed. Within 3 minutes of the drug being added the furrow would begin to regress in all cells measured (figure 19). Both sides of the furrow were

affected equally. Once 20 mM was washed out from the cell the furrow would return to the original length and continue to slowly ingress.

Table 12. Effect of 20 mM BDM on bivalent length, cell length, and furrow movement.

	20 mM BDM Treatment		
	Average Change	*n value*	*Range*
Bivalent length*	+21% ± 0.16% (SE)	6 bivalents	18 – 25%
Cell length*	-5% ± 0.013% (SE)	7 cells	4 - 7%
Furrow movement*	+15% ± 0. 84% (SE)	7 cells	8 – 32%
* $p \leq 0.05$			

BDM had an effect on bivalent reorientations in the cell. Within 15 minutes of washing out 20 mM BDM a bivalent would reorient so that both kinetochores were facing the same pole (figure 20). This occurred in 4 cells treated with 20 mM BDM and 5 bivalent reorientations were observed. In one cell, 2 bivalents reoriented to the same pole within a minute of each other. This corresponds to a bivalent reorientation every 30.2 minutes. In all 10 cells, no univalent excursions were observed.

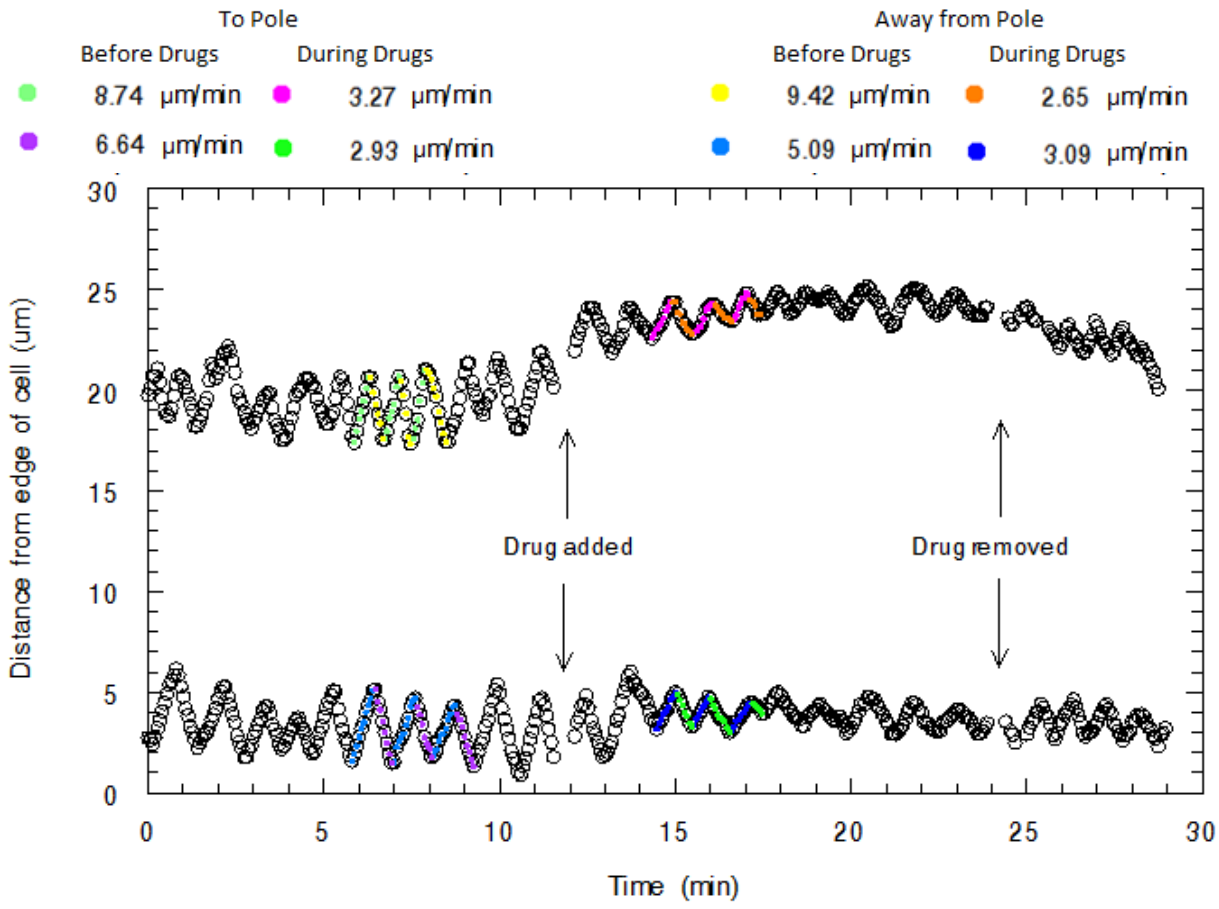

Figure 18. *Movement of a single bivalent in a primary spermatocyte treated with 20 mM BDM. The velocity and amplitude both decreased 2 minutes after BDM was added. The length of the bivalent continued to increase the longer BDM was present. Once washout occurred the length of the bivalent began to decrease and oscillation velocity and amplitude started to recover.*

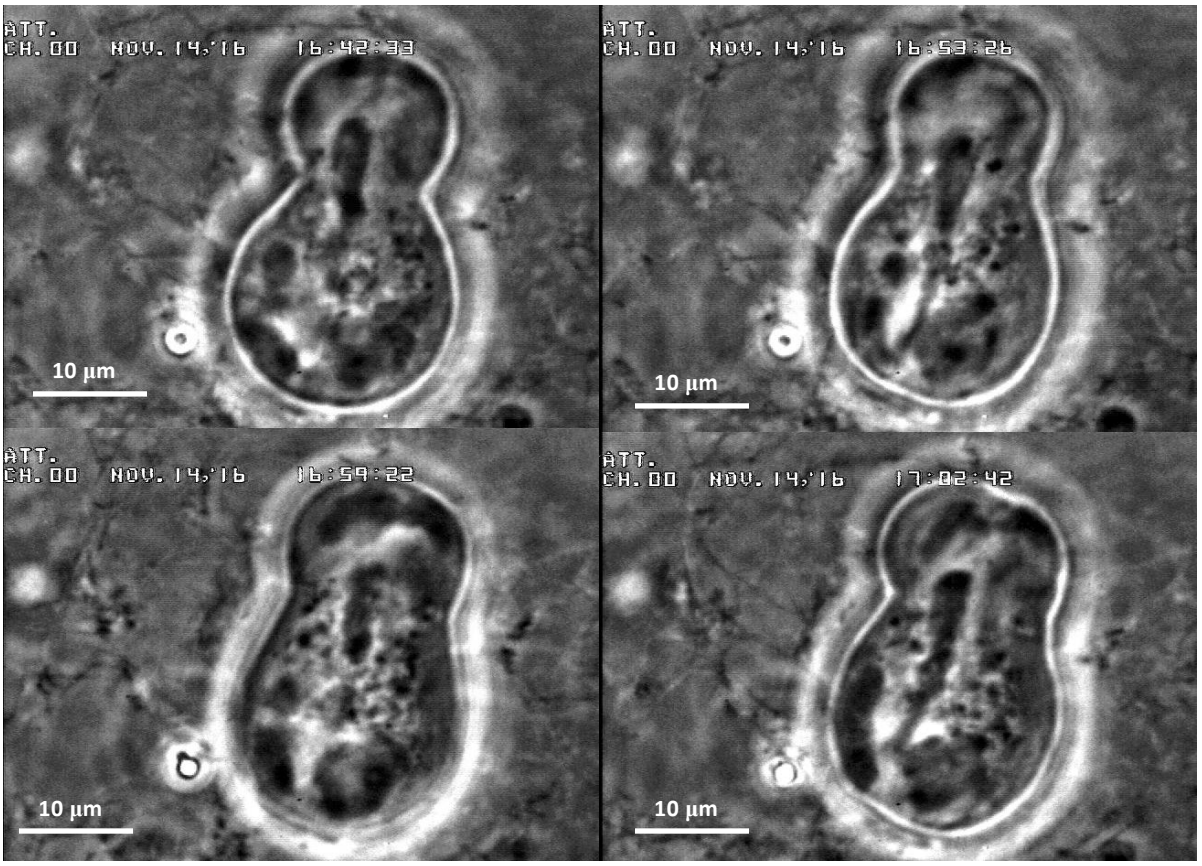

Figure 19. *Pictures from a video sequence of a primary spermatocyte being treated with 20 mM BDM at 16:47:18. The furrow begins to regress within 3 minutes of BDM being added. Once BDM was washed out at 16:59:03 the furrow begins to ingress and recover.*

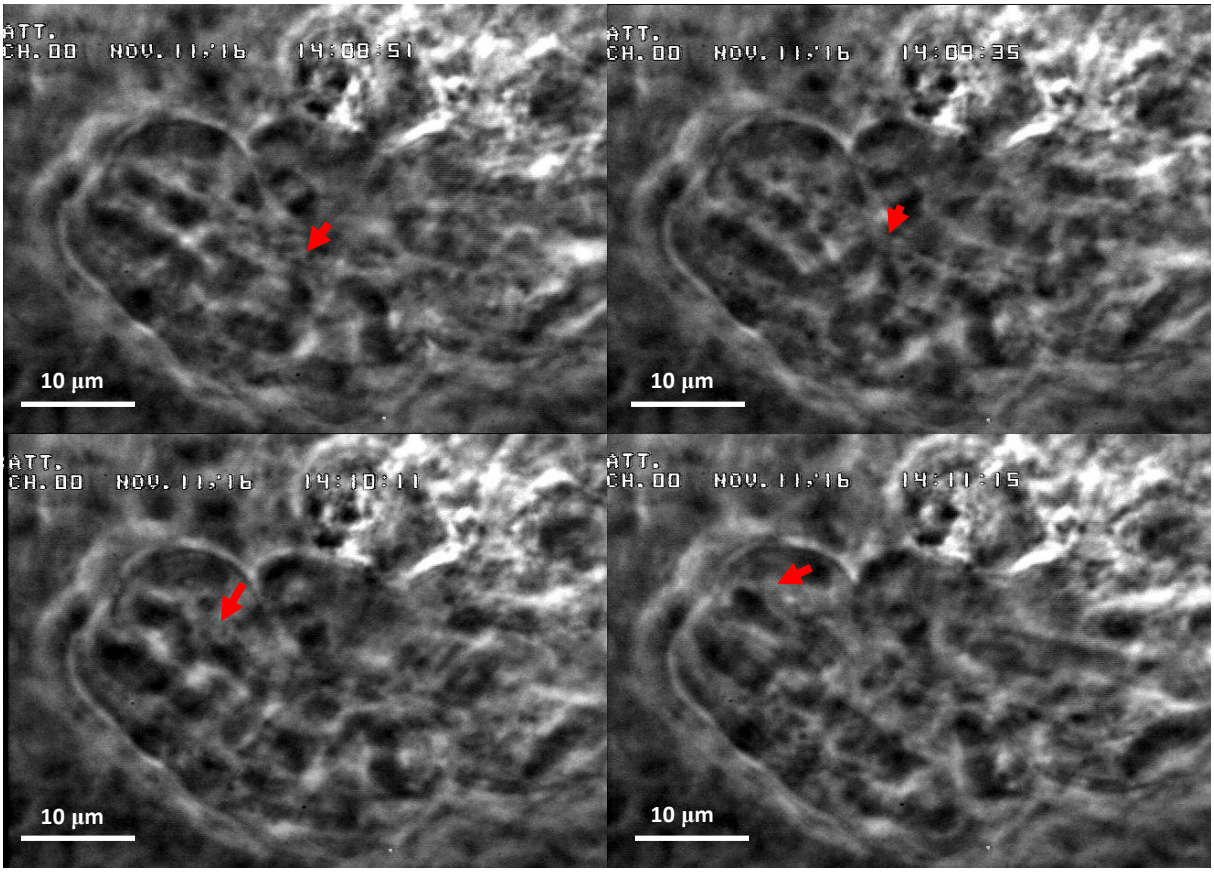

Figure 20. *Pictures from a video sequence of a primary spermatocyte treated with 20* m*M BDM at 13:42:52 for 10 minutes before the drug was washed out. Shortly after washout at 13:54:56, a bivalent reorientation could be observed. The kinetochore facing the right pole reorients so that it is facing the left pole in the bottom cell. The red arrow is pointing out the kinetochore reorienting to face opposite pole.*

Effects of Protein Phosphatase 1 Inhibition using Calyculin A

To further investigate the role of myosin phosphorylation in bivalent oscillations in primary spermatocytes I treated the cells with phosphatase inhibitor. Calyculin A is a naturally occurring compound that specifically targets Ser/Thr protein phosphatase 1 (Suganuma et al., 1990). When I treated *M. ehrenbergii* primary spermatocytes with calyculin A during prometaphase there was an increase in the velocity of the kinetochore movement towards and away from the pole (figure 21). An increase was also observed in the amplitude, and there were observable effects in the period (table 12). The effects were observed within 2 minutes of calyculin A treatment. Bivalents within a single cell were not affected equally. Some bivalent speeds increased so much that the spindle would collapse under the force. This was common at 10, 5, and 2.5 nM concentrations but did not occur with 1 nM calyculin A.

Table 12. Effects of calyculin A on bivalent oscillations in prometaphase.

	Calyculin A Treatment		
	Velocity	*Amplitude*	*Period*
Average Change	Towards pole: + 89% ± 16.8% (SE)*** Away from Pole: +85% ± 18.8 (SE)***	+64% ± 19 (SE)**	Decreased 19.5% ± 1.5 (SE)**
Number of Kinetochores*	12	12	28
Range	Towards pole: 31-189% Away from pole: 26 – 222%	23 – 234%	Decrease: 14 – 57%
Concentration	1 nM	1 nM	1, 2.5, 5, 10 nM
*represents n value, ** $p \leq 0.05$, *** $p \leq 0.01$			

The velocity of the movement towards or away from the pole was affected in a similar manner. Calyculin A did not affect one direction more compared to the other and the distribution was fairly even with the majority of velocities increasing by 81-90%.

Similar to BDM, there was an observed effect on the period of bivalent oscillations. Calyculin A treatment however only caused a decrease in the period and not an increase. The period of kinetochores oscillations was affected at all concentrations of calyculin A, but the 1 nM concentration had a smaller effect (table 13).

Table 13. Effect of Calyculin A on oscillation periods in prometaphase.

Treatment	Number of cells	Number of Kinetochores			
		Total	Increased	Decreased	No Change
10 nM calyculin A	3	6	0	6	0
5 nM calyculin A	5	10	0	10	0
2.5 nM calyculin A	5	9	0	7	2
1 nM calyculin A	5	10	0	5	5

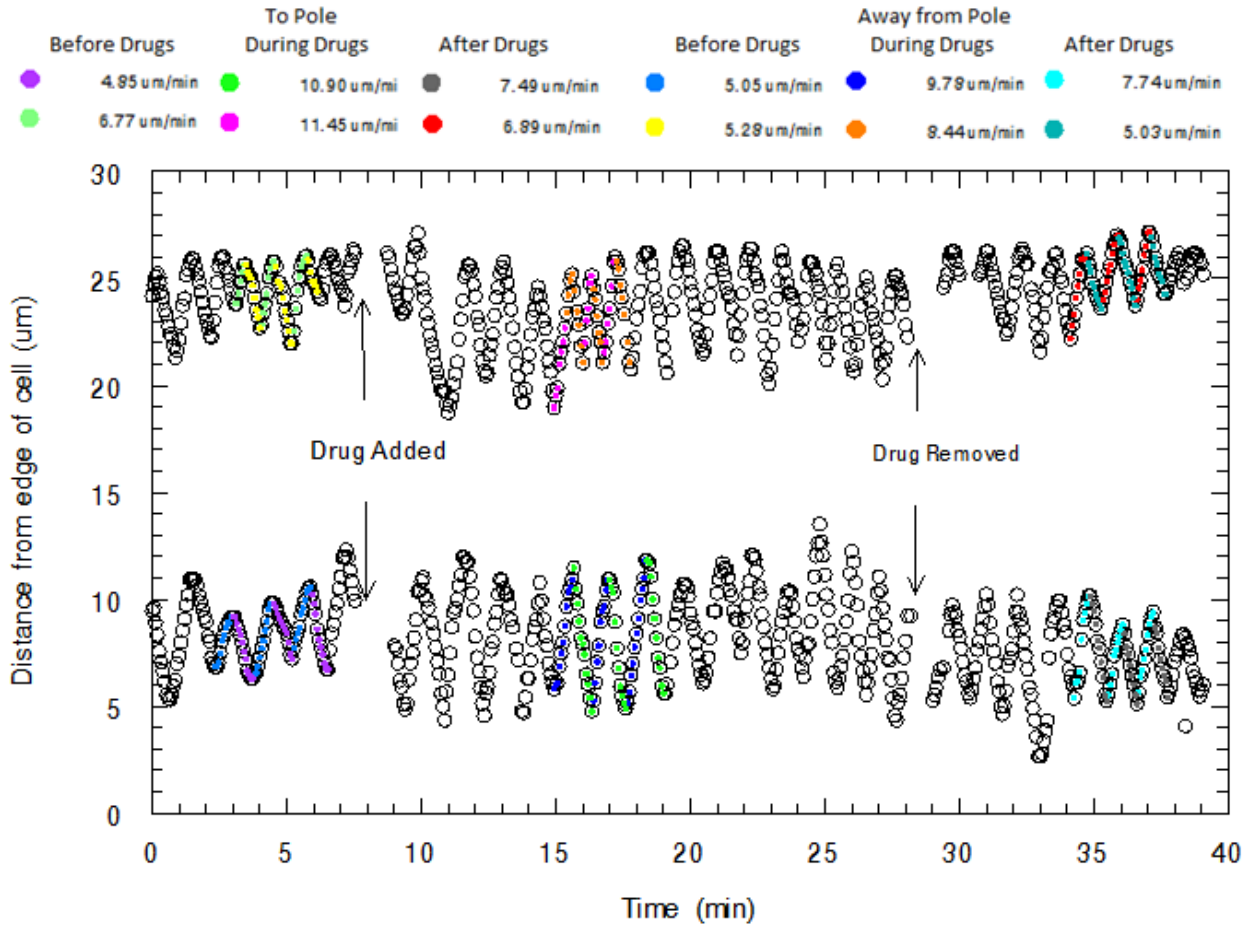

Figure 21. *Movement of a single bivalent in a primary spermatocyte treated with 1 nM calyculin A for 19 minutes before wash out. The velocity and amplitude increased within 2 minutes of calyculin A while the period decreased. The velocity, amplitude and period begin to recover 4 minutes after 1 nM calyculin A is washed out.*

Recovery was only observed when 1 nM calyculin A was washed out. In all of the primary spermatocytes treated with higher concentrations of calyculin A, the spindle collapsed after being exposed to calyculin A for 5 minutes or more. With 1 nM calyculin A, the spindle did not collapse and recovery data could be obtained (table 14).

Table 14. Recovery of bivalent oscillations after 1 nM calyculin A washout.

			Number of Kinetochore		
	Number of cells	Average Recovery	Total	Recovery	No Recovery
Velocity (compared to original value)	5	Towards pole: 50% higher Away from pole: 44% higher	10	Towards pole: 8 Away from pole: 10	Towards pole: 2 Away from pole: 0
Amplitude	5	43% higher	10	10	0
Period	3	97%	5	5	0

The length of the bivalents in the cells treated with calyculin A was not affected (excluding 1 nM treatment), but in all concentrations the length of the cell was (table 15). Shortly after the addition of calyculin A the length of the cell would increase. This would continue to occur until the spindle would collapse in higher concentrations causing the length of the cell to decrease as it died. Upon the addition of calyculin A, the furrow of the cell would tighten within 2 minutes (figure 22). This indicates that calyculin A is indeed targeting myosin phosphorylation. The furrow continued to ingress until the furrow collapsed in higher calyculin A concentrations. Once the spindle collapsed the furrow was completely lost and the cell became round.

Table 15. Effect of calyculin A on bivalent length, cell length, and furrow movement.

	Calyculin A Treatment		
	Average Change	*n value*	*Range*
Bivalent length*	-18% ± 0.47 (SE)	5 bivalents	13- 29%
Cell length**	+6% ± 0.05% (SE)	10 cells	3 – 12%
Furrow movement**	-16% ± 0.5 (SE)	9 cells	8 – 32%
* $p \leq 0.01$, ** $p \leq 0.05$			

When I treated primary spermatocytes with calyculin A the spindle collapsed after 5 minutes (figure 23) of treatments except in the 1 nM condition. A kinetochore would pull away from one pole and clump up in the opposite. In 7 of 13 cells, a univalent movement was observed in the same direction as the kinetochore movement. The 7 univalent excursions, observed in 7 cells corresponded to a univalent movement every 16.3 minutes. No bivalent reorientations were observed, all bivalents that did move would clump to the same pole. Both kinetochores would not reorient to face the same pole.

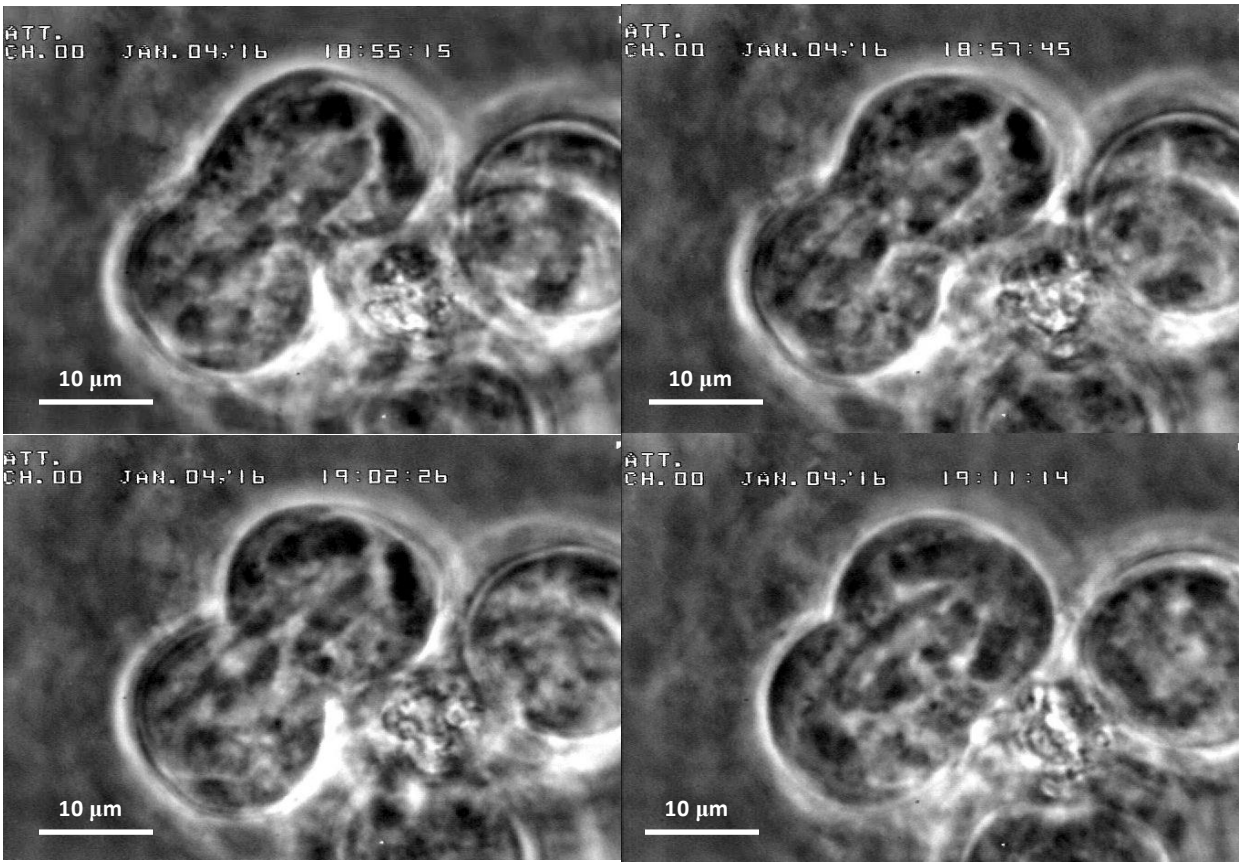

Figure 22. *Pictures from a video sequence of a primary spermatocyte treated with 2.5 nM Calyculin A at 18:55:37. The furrow begins to ingress 2 minutes after the addition of calyculin A. Washout occurs at to primary spermatocytes leads to furrow regression. This would occur about 2 minutes after 2.5 nM calyculin A was added. The spindle begins to collapse at 19:11:05 and the furrow begins to regress.*

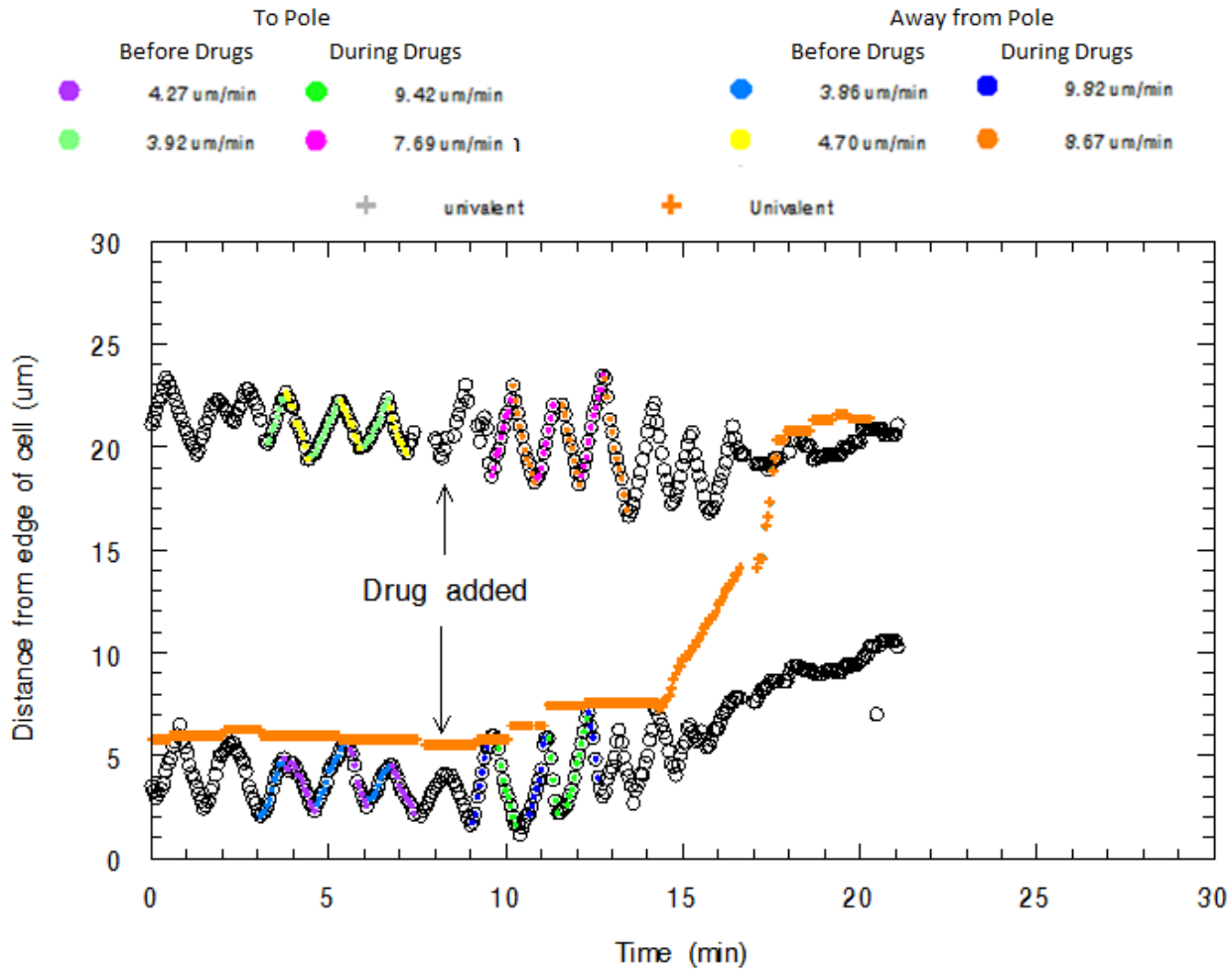

Figure 23. *Primary spermatocytes were treated with 2.5 nM Calyculin A. Within a minute of calyculin A treatment the velocity and amplitude increased. The spindle collapsed after 6 minutes of exposure. When the spindle collapsed the bottom kinetochore pulled away from the pole and clumped in the opposite pole. At the same time, a univalent moved in the same direction at the kinetochore pulling away from the pole.*

Okadaic acid as a Control for Calyculin A Effects on Bivalent Oscillations

Calyculin A is a potent Ser/Thr protein phosphatase inhibitor but can affect both PP1 and PP2A (Ishihara et al., 1989). In order to determine if the results I obtained are a result of PP1 inhibition and not PP2A, I used okadaic acid. Okadaic acid is a toxin that targets Ser/Thr phosphatases (Dounay & Forsyth, 2002). Okadaic acid can inhibit the activity of both PP1 and PP2A but has a much higher affinity for PP2A. Okadaic acid (5 and 10 nM) had no major effects on bivalent oscillations in *M. ehrenbergii* primary spermatocytes (table 16, figure 24). Therefore the effects observed during calyculin A treatment are a result of protein phosphatase 1 inhibition and not protein phosphatase 2A. Primary spermatocytes treated with okadaic acid also had no observable effects in bivalent length, cell length, furrow movement and the occurrence of bivalent reorientations and univalent excursions.

Table 16. Effect of 5 and 10 nM okadaic acid on bivalent oscillations.

Treatment	Parameter	Number of cells	Number of Kinetochores			
			Total	Increase	Decrease	No change
Okadaic acid	Velocity	7	14	1	0	13
	Amplitude			0	0	14
	Period			0	0	14

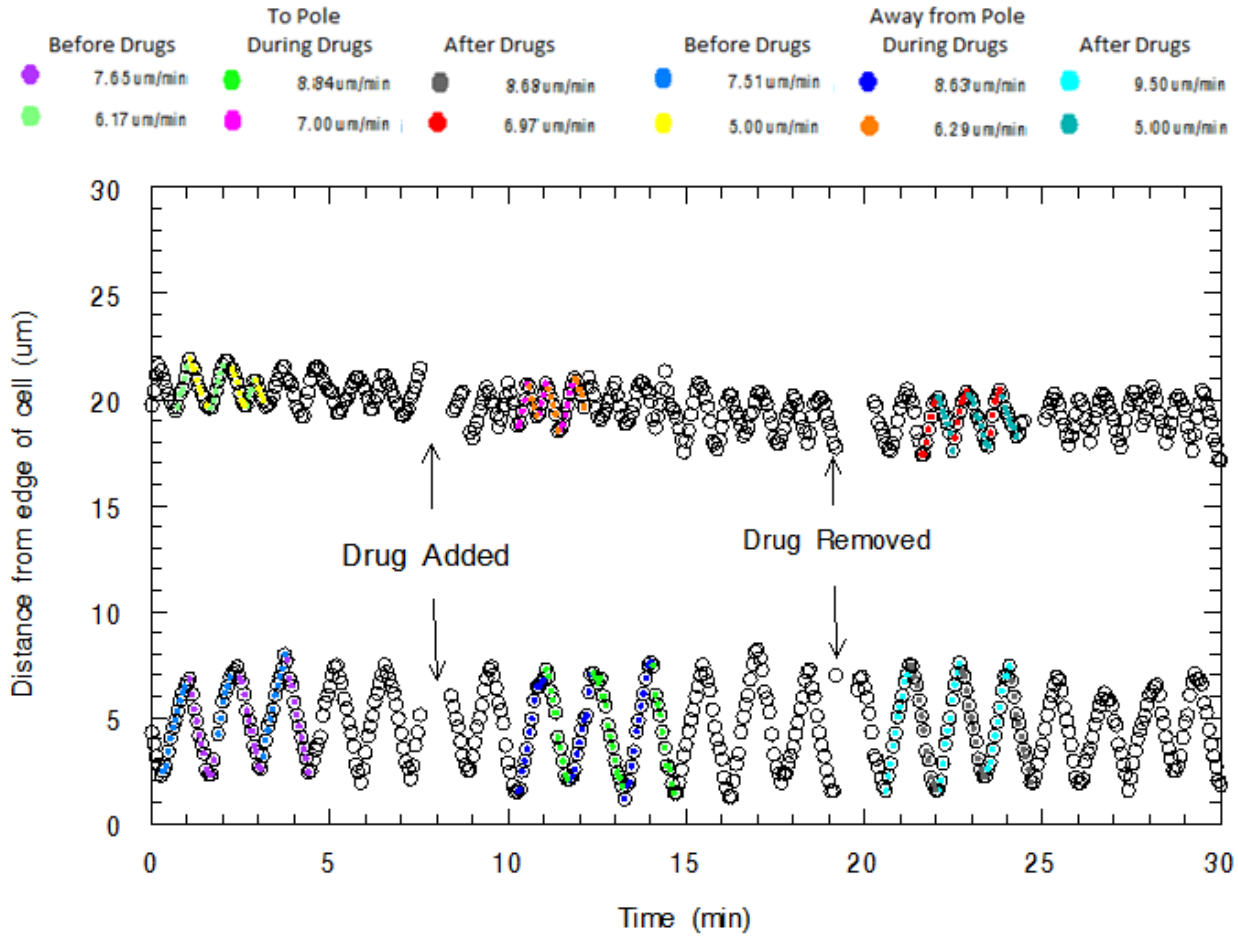

Figure 24. *Movement of a single bivalent in a primary spermatocyte obtained on February 1st, 2017 treated with 10 nM okadaic acid. No significant change is observed in the velocity, amplitude or period of kinetochore oscillations.*

Table 17. *Summary of the effects of myosin inhibitors and enhancers on all characteristics measured.*

	Effects caused by drugs					
	Myosin Inhibitors				**Myosin Activators**	
	ML-7	**Y-27632**	**H-7**	**BDM**	**Calyculin A**	**Okadaic Acid**
Velocity	Decreased	Decreased	No Change	Decreased	Increased	No Change
Amplitude	Decreased	Decreased	No Change	Decreased	Increased	No Change
Period	No Change	No Change	No Change	Increased & Decreased	Decreased	No Change
Bivalent length	No Change	No Change	No Change	Increased	No Change	No Change
Cell length	Decreased	Decreased	No Change	Decreased	Increased	No Change
Furrow	Regress	Regress	No Change	Regress	Ingress	No Change
Univalent Movement	No Change	Decreased	No Change	No Change	Increased	No Change
Bivalent Reorientation	No Change	Decreased	No Change	Increased	No Change	No Change

Discussion

General Discussion

The purpose of my thesis is to better understand if and how myosin is involved in the force production allowing for the movement of chromosomes during prometaphase I in meiosis. I used a pharmacological approach in order to manipulate chromosome movement in primary spermatocytes of adult *Mesostoma ehrenbergii.* Drugs were used that target myosin in either a direct or indirect manner. The activity of myosin was then either inhibited or increased and results were observed. Since *M. ehrenbergii* have 3 large bivalents that have regular oscillations for 1-2 hours, they make an ideal organism for studying movement during prometaphase. In studying the role of myosin, I followed the velocity, amplitude and period of bivalent oscillations. I hypothesized that myosin contributes to the force production required for chromosome oscillations to occur in *Mesostoma ehrenbergii* primary spermatocytes and that there are redundant pathways. Confirming my hypothesis, results showed that multiple phosphorylation pathways are required to activate spindle myosin and that myosin is not the only force contributor.

Pharmacological Studies Targeting Myosin

In my thesis, I used different drugs that inhibit and enhance the activity of myosin. The experiments suggest that myosin is involved in force production in *Mesostoma* spermatocytes. When the activity of myosin is inhibited the movements of the kinetochores towards and away from the pole decreased, as well as the amplitude.

Enhancing the activity of myosin causes the velocity and amplitude of the oscillations to increase. Another conclusion that can be made is that there are redundant pathways to phosphorylate myosin within the spindle. The period of the oscillation is only affected when total myosin is either inhibited or enhanced. When only single phosphorylation pathways were perturbed, the period was not altered. When two phosphorylation pathways were inhibited, bivalent oscillations were not completely stopped indicating that additional phosphorylation pathways may contribute to the movement. In order to confirm this assumption, additional phosphorylation pathways will need to be inhibited and observed for effects on chromosome oscillations. Additional phosphorylation pathways can also be determined by challenging the inhibitors with calyculin A to see if movement can be increased. In addition, myosin is not the only force contributor for chromosome oscillations during prometaphase in primary spermatocytes. When total myosin was inhibited using BDM, bivalent oscillations still persisted. This indicates that in addition to myosin, there are additional mechanisms that contribute to bivalent oscillations.

Bivalent Oscillations

Through my inhibition studies, both MLCK and ROCK phosphorylation pathways were inhibited. ML-7 was used to inhibit MLCK and Y-27632 was used to inhibit ROCK. Individually both drugs elicited similar results. Shortly after the addition of the drug, the velocity of chromosome movement towards the pole and away from the pole both decreased, as well as the amplitude. In order to determine whether MLCK and ROCK are the only two pathways responsible for the phosphorylation of myosin in the spindle, both ML-7 and Y-27632 were added together. The velocity of the movement towards

the pole as well as away from the pole decreased but none of the chromosomes stopped or almost stopped movement. This suggests that the two kinases targeted with ML-7 and Y-27632 are not the only pathways that are responsible for myosin phosphorylation. Since the movement did not completely stop, this suggests that there are still other phosphorylation pathways to activate some of the spindle myosin.

For both of the kinase inhibition scenarios, the bivalent shifted towards a single pole while the drug was present. In the majority of the cells treated with ML-7 and Y-27632 separately, the position of both the bivalent would shift towards a single pole. This could indicate that in a single bivalent, myosin may have a larger impact in producing force in a single direction. In unpublished experiments by Fegaras and Forer, microtubules were removed from an *M. ehrenbergii* primary spermatocyte using nocodazole. Upon the removal of the microtubules, all of the chromosomes moved towards a single pole except in a few cells. The authors suggested that the fibres in the microtubule spindle or matrix spindle are asymmetric. Based on my experiments, this would suggest that the force from myosin may be denser on one side and that the force of myosin may have a larger impact in one direction. With the microtubules in the cell gone, there may not be a sufficient amount of force to counteract the force of myosin in this single direction causing the chromosomes to go to the single pole. In the case of the cells I treated, the decreased force being exerted by myosin would no longer be balanced by the force of microtubules causing the bivalents to move in the opposite direction.

These results are similar to those that were obtained in crane-fly primary spermatocytes by Sheykhani, Shirodkar, and Forer (2013). The addition of ML-7, as

well as Y-27632, caused the chromosomes to stop or slow during anaphase movement. They then challenged this result by adding calyculin A in the presence of either ML-7 or Y-27632. Results showed that the previously slowed chromosomes were able to accelerate once calyculin A was added to the cell. This shows that more than one phosphorylation pathway is used to activate myosin within the spindle to contribute to force production. In addition to this experiment, calyculin A was added in the presence of staurosporine and there was no increase in chromosome speed. Staurosporine is a general kinase inhibitor that is isolated from *Streptomyces staurosporeus* (Omura et al., 1977). This again, further supports that there are other phosphorylation pathways that can contribute to force production by spindle myosin. This is similar to what occurs in smooth muscle cells, and the many myosin phosphorylation pathways have been well studied (Amano et al., 1996, Shabir et al., 2004).

A potential candidate for an additional phosphorylation pathway could be citron kinase that was previously discussed in the introduction. This kinase has been studied in *Drosophila melanogaster* and has been found to play a specific role in the final steps of cytokinesis (Naim et al., 2004). Another potential candidate could be ILK, which has been found to have implications during mitosis including microtubule dynamics (Fielding et al., 2008). Blocking both citron kinase and integrin-linked kinase in addition to MLCK and ROCK may lead to similar results obtained when the cell was treated with staurosporine. If the addition of calyculin A still causes an increase in chromosome movement then there may be more pathways involved in phosphorylation. This makes the assumption that targeting different kinase that effect myosin phosphorylation only reduces it and does not completely diminish it.

In addition to inhibiting upstream proteins that lead to the phosphorylation of myosin, a drug was used that directly inhibits the function of myosin. BDM binds to myosin and does not affect the ability of different kinase to phosphorylate the myosin regulatory light chain or affect the ATPase ability of the myosin head. Instead, this drug acts to stabilize the ADP and inorganic phosphate complex, preventing the power stroke from occurring (Forer and Fabian, 2005). I used this drug in order to completely inhibit spindle myosin involved in the movement of chromosome oscillations. If myosin is the only component responsible for chromosome movement then, chromosome movements should completely stop. This, however, was not what was observed during experiments. The movement of the chromosomes was affected to a larger degree compared to ML-7 and Y-27632, but the movement of the chromosomes was never completely stopped. This suggests that myosin is not the only force producer within the spindle. The reason for the chromosomes not completely stopping could be due to a number of reasons. Some of the motion could be attributed to elastic recoil as discussed by Nicklas and Staehly (1967). Another potential candidate that could be attributing to force could be microtubules and its associated motor proteins.

In two of the bivalents treated with BDM, however, the movement did seem to almost completely stop to the point where the bivalents just vibrated in place. Although there are many different mechanisms that can contribute to chromosome movement, some bivalents may rely more heavily on one mechanism compared to the other. This could depend on the conditions present in the cell when the spindle is being formed. If a portion of the cell has a higher concentration of myosin when the spindle apparatus is being formed, this may cause this portion to rely more heavily on this protein for

movement. In some studies, the addition of drugs may slow the movement of some chromosomes, while completely stopping the movement of others (Fabian & Forer, 2005; Fabian et al., 2007; Silverman-Gavrila & Forer, 2001).

When ML-7 and Y-27632 were added individually as well as together to *M. ehrenbergii* primary spermatocytes, there was no observed change in the period of the oscillations. This, however, is not the case when BDM was added to the cells. When total spindle myosin was inhibited by the addition of BDM there was a noticeable change in the period of oscillations, with some increasing and others decreasing. I believe that the reason for this sudden change in period is due to the fact that all myosin was being inhibited instead of only some phosphorylation pathways. Since some myosin was still able to function within the spindle, the period was still able to be maintained.

M. ehrenbergii primary spermatocytes were exposed to calyculin A which blocks the dephosphorylation of the myosin regulatory light chain. With the addition of calyculin A, the velocity of the chromosomes increased at all concentrations used as well as the amplitude of the oscillations. This further supports that spindle myosin is involved in producing the force that contributes to chromosome movement. As this drug causes the myosin to be hyperphosphorylated this would increase the activity of myosin causing the chromosomes to oscillate faster.

In the higher concentrations of calyculin A, however, the force of myosin seemed to exceed that of the underlying spindle interacting with the chromosomes. This caused the underlying structure to collapse and the chromosomes to move towards a single

pole. These results are very similar to those obtained in the previously mentioned unpublished study which used nocodazole to depolymerize microtubules in the cell. This further supports the idea that the force of myosin may be more dominant in one direction of the cell compared to the other.

The structure that opposes the force of myosin may or may not be the microtubules within the cell. Many other candidates such as megator (Qi et al., 2004), skeletor (Walker et al., 2000), EAST (Qi et al., 2005) and chromator (Rath et al., 2004) could act as an opposing force. Each of these proteins has been found to localize in the spindle or create their own spindle. Skeletor as an example may interact with myosin within the spindle and act as an opposing or regulating force. When skeletor redistributes to the cytoplasm it forms a spindle-like structure that lines up with the spindle formed by microtubules (Walker et al., 2000). Since these two structures overlap it would be difficult using staining methods to determine which spindle structure myosin is interacting with. Further studies need to be conducted in order to determine what proteins myosin is interacting with. For example, a study can be done where the spindle formed by skeletor is removed to see if they same results occur.

To further support the conclusion that all spindle myosin needs to be affected before a change in the period can be observed are the cells that were treated with calyculin A. The period of the oscillations decreased becoming more frequent with the addition of calyculin A. Since there is only one protein phosphatase 1 that is responsible for the dephosphorylation of the myosin regulatory light chain, there is only one pathway to target. Adding calyculin A to the cell effectively blocks this single pathway, causing a change in the period of the cell. From this, I can suggest that spindle myosin helps to

regulate the period of oscillations during prometaphase in *M. ehrenbergii* primary spermatocytes.

Together these drugs support the conclusion that spindle myosin is involved in chromosome oscillations in *M. ehrenbergii* primary spermatocytes. Previous studies have found that myosin is present in the spindle and that pharmacological manipulation of myosin activity alters chromosome movements. In previous studies, myosin has been identified in the spindle in many different cell types as reviewed in Forer et al. (2003), and both phosphorylated and unphosphorylated myosin, were localized to the spindle fibres. Examples include crane-fly spermatocytes (Silverman-Gavrila and Forer, 2001 and 2003; Fabien et al., 2005; Fabien et al., 2007; Sheykhani, Shirodkar & Forer, 2013), human mitotic spindles (Fujiwara & Pollard, 1976), PtK cell spindles (Sanger et al., 1989; Snyder et al., 2010) and sand-dollar spindles (Uehara et al., 2008).

In crane-fly spermatocytes as well as locust spermatocytes the addition of myosin inhibitors altered chromosome movements causing them to stop or slow (Silverman-Gavrila & Forer, 2001 and 2003; Fabian et al., 2005; Fabian & Forer, 2007; Fabian et al., 2007). Similar results were also found when myosin inhibitors were added to PtK cells (Snyder et al., 2010). In rat kidney cells, when the activity of myosin was blocked, anaphase was unable to occur (Komatsu et al., 2000). In addition to being responsible for chromosome movements during anaphase, myosin has also been implicated in the proper formation of the spindle. This was observed in sea urchin zygotes as well and NRK cells when myosin was inhibited, causing the organization of microtubules to be altered (Mabuchi et al., 1990; Fishkind et al., 1991).

Precocious Cleavage Furrow

In *M. ehrenbergii* primary spermatocytes a precocious cleavage furrow is formed at the beginning of prometaphase. This furrow remains present and slowly ingresses as the cell progresses towards cytokinesis before completely cleaving. The furrow is a contractile ring that is composed of filamentous actin and myosin. Together these two proteins work together to form a furrow around the cell that leads to the partitioning of the two newly formed cells at the end of cytokinesis (Miller, 2011). If the drugs I am using in my study are targeting myosin activity then effects on the furrow should be observed. Myosin inhibitors and enhancers both affected the precocious cleavage furrow causing regression and ingression respectively.

When ML-7 and Y-27632 were added to the primary spermatocytes either together or individually, there was a visible effect on the precocious cleavage furrows. Shortly after the drugs were added the furrows would begin to regress. Similar results were also obtained when primary spermatocytes were exposed to BDM. The degree of regression varied considerably and was not consistent even within the same drug treatment. When Y-27632 was added to the cell, in a majority of the cells only 1 side of the furrow regressed. This could, however, be explained by the retainment of phosphorylated myosin within the furrow. In a study by Miyauchi and colleagues (2006), HeLa cells were transfected with 3 different GFP-tagged recombinant MRLCs. All forms of the myosin regulatory light chain were recruited to the furrow whether they could be phosphorylated or not. However, once cytokinesis began only phosphorylatable forms of the myosin regulatory light chain were retained in the furrow, while the other forms were removed. The cells ability to select for myosin that is able to be phosphorylated

may contribute to the fact that furrow regression was not consistent or one-sided in the cells. The cells may have been actively removing myosin which could not be phosphorylated at different rates causing the furrow to regress in a one-sided manner or less compared to other cells. Many experiments have been conducted that show that the addition of different myosin inhibitors causes the furrows to regress during cytokinesis. An example of this would be in HeLa cells that were treated with a myosin inhibitor (Asano, Hamao & Hosoya, 2009; Kondo et al., 2011). HeLa cells were treated with a myosin inhibitor and effect on the furrow were observed. The addition of the drug caused there to be a delay in furrow ingression and caused furrows that were already ingressing to slow down considerably. Other examples have been seen in U251 cells treated with Y-27632 (Kosako et al., 2000) and sea urchin eggs (Uehara, Hosoya & Mabuchi, 2008).

The addition of calyculin A had the opposite effect on the precious cleavage furrow. The primary spermatocytes that were exposed to calyculin A showed a further ingression of their furrows shortly after the addition of drug. Since this drug targets protein phosphatase 1, this would cause myosin to become hyperphosphorylated, increasing the activity and causing the furrow to ingress further. The effects of calyculin A on cleavage furrows in different cell types has been well studied. When unfertilized sea urchin eggs are treated with calyculin A, a cleavage furrow is induced (Goda, Inoue & Mabuchi, 2009). These cleavage furrows were irregular as they do not always form in the middle of the cell and can form in different planes. This was also observed when newt eggs were treated with calyculin A causing the formation of multiple furrows along the length of the cell (Sawai, 1997).

Univalent and Bivalent Movements

Throughout prometaphase, univalent excursions and bivalent reorientations can be observed until the cell is ready to proceed into anaphase. As the chromosomes move to the opposite poles the furrow responds by moving towards the pole with fewer chromosomes. In studying the role of myosin, I found that a specific myosin phosphorylation pathway is involved in univalent excursions and bivalent movements. Univalent movements decreased only when the Rho-kinase phosphorylation pathway was inhibited. This suggests that this pathway may be involved in these movements. This myosin phosphorylation pathway may not be the only mechanism that contributes to these movements. Univalent movements and bivalent reorientations were observed in the presence of Y-27632, and were not affected by BDM treatment. Since BDM inhibits all myosin, the occurrence of univalent movements would be expected to decrease based on Y-27632 results but was not observed. This suggests that Rho kinase may be required to activate a different pathway and that the activity of myosin may be indirectly involved.

When cells were treated with Y-27632 the number of univalent excursions and bivalent reorientations was decreased compared to the 1 every 21.5 minutes and 1 every 50 minutes respectively obtained in previous studies (Ferraro-Gideon, Hoang & Forer, 2014). When univalent movements or bivalent reorientations did occur in the presence of drug the cell was no longer able to proceed into anaphase. This suggests that the movement was either not recognized by the cell or was not supposed to happen causing there to be an error in chromosome movements. The same univalent or bivalents were not observed to switch back to the original pole. In the case where the

same univalent was observed moving back to the original pole, the cell was able to complete anaphase. This suggests that the error that occurred with the drug present was corrected. Since only a decrease in these movements was observed when Rho-kinase was inhibited this may suggest that it is involved in this process and proper functioning is required for these movements.

In cells that were treated with BDM, there was an increase in the number of bivalent reorientations. These reorientations did not occur in the presence of the drug but rather occurred once the drug was washed out. Although it seems that these movements were increased, they may have been actively suppressed in the presence of the drug and could only occur once the drug was moved. This could also be a result of rho-kinase pathways not properly functioning. With BDM removed from the cell, the amount of myosin phosphorylation by Rho-kinase would increase which could be why the bivalent reorientations increased. This agrees with cells that were treated with Y-27632 as the pathway was inhibited so were the number of bivalent reorientations decreased. While BDM was present there was no observed decrease in univalent excursions or bivalent reorientations which was expected based on Y-27632 experiments. Although the Rho-kinase pathway may be involved, myosin may indirectly contribute to the movement. This would explain why no decreases were observed when total myosin was inhibited and not a phosphorylation pathway.

In cells treated with calyculin A, there was an observed increase in the number of univalent excursions. Although these movements did occur in the presence of the drug, they also occurred once the spindle seemed to collapse. I suggest that the increase in univalent excursions was partially due to the increased activity of myosin as well as the

collapse of the spindle. With the addition of calyculin A, all myosin phosphorylation pathways would increase allowing for hyperphosphorylation. This could indirectly lead to the increased activity of Rho-kinase, which could increase the number of univalent excursions. Another conclusion could be based on the number of fibres that are present in each half of the cell. In an unpublished study by Fegaras and Forer where nocodazole was used to depolymerize microtubules in the cell, all of the chromosomes moved towards a single pole. The experimenters suggested that this may be due to more fibres present in one half of the cell compared to the other. If this conclusion is accurate then this would account for the increased number of univalent movements once the spindle seemed to collapse. There could theoretically be more myosin in one half of the cell compared to the other causing the chromosomes to be pulled to one side of the cell once there was no opposing force. The increased number of bivalent reorientations may not have been observed because of the spindle collapsing. These conclusions agree with Y-27632 results but further studies need to be conducted in order to fully understand the relationship between myosin activity, univalent excursions, and bivalent reorientations.

Pharmacological Studies Conclusion

The main conclusion that can be drawn from my experiments is that myosin does play a role in contributing to force production that allows for the movement of chromosomes. I can also conclude that there are redundant pathways leading to the phosphorylation of the myosin regulatory light chain. In addition to having redundant pathways for phosphorylation, there are also additional forces on the chromosomes allowing for movement. The additional force may be produced by but is not limited to

microtubules, skeletor, and megator. The above conclusions provide evidence to support my hypothesis. Myosin phosphorylation in *M. ehrenbergii* primary spermatocytes produces force that contributes to the velocity, amplitude and period of oscillations. The period of the oscillations, however, is only affected if total myosin is inhibited or activated and not only 1 or 2 phosphorylation pathways.

My experiments suggest that activity of myosin affects the ingression and regression of the precocious cleavage furrow. It does not, however, seems to play a role in the position of the furrow along the length of the cell as normal movement during univalent movement and bivalent reorientations were observed when drugs were present. A single phosphorylation pathway did seem to implicated in univalent excursions and bivalent reorientations. Based on my experiments, I suggest that the Rho-kinase phosphorylation pathway may have a role in these events occurring. I do not believe however that this is the only pathway as the effects of BDM and calyculin A were not completely conclusive. I suggest that there are redundant pathways to allow for these events to occur and that further studies need to be done to fully understand these movements as well as their purpose.

Based on my studies I suggest that the force of myosin in the cell in concentrated more in one direction compared to the other. This conclusion is based on the fact that bivalent shifted towards a single pole as well as univalents and bivalents moving towards a single pole once the underlying spindle structure has collapsed. This conclusion, however, needs to be further supported by staining and additional pharmacological studies.

Future Directions

To further support the conclusions that I have drawn additional studies need to be carried out. I would like to do additional inhibitions studies to determine other myosin phosphorylation pathways that are involved in chromosome movements such as citron kinase and integrin-linked kinase. Potential drugs include 5-{5-[(2S)-2-amino-3-phenylpropoxy]pyridin-3-yl}-3-methyl-1H-indazole (A-674563) and QLT0267 which inhibit citron kinase and integrin-linked kinase respectively (Davis et al., 2011; Fielding et al., 2008). If citron kinase and integrin-linked kinase are involved in bivalent oscillations, I would then like to use QLT0267, A-674563, ML-7 and Y-27632 together to see if movement can be completely stopped. In addition, I would also like to challenge the inhibitors by adding calyculin A to determine if there are more phosphorylation pathways.

Once those have been completed I would then like to stain for total myosin within the spindle as well as monophosphorylated and diphosphorylated myosin. The stains are MY-21 (mouse IgM antibody) for total myosin regulatory light chain (MRLC) tubulin staining, Phospho-Myosin Light Chain 2 (Ser 19) Mouse IgG antibody for mono-phosphorylated myosin regulatory light chain and Phospho-Myosin Light Chain 2 (Thr18/Ser19) rabbit antibody for bi-phosphorylated myosin regulatory light chain. To act as a control, tubulin will also be stained for using YL1/2 rat monoclonal antibody.

Three different types of fluorescent staining for myosin are going to be used because I want to determine the location(s) of myosin during prometaphase in *M.*

ehrenbergii spermatocytes. This will also allow me to see exactly where in the cell myosin is being affected by the drugs. If results show that there is less myosin phosphorylation associated with the spindle then this gives me a good indication that the decreases in velocity were a result of the inhibition of myosin. In cells that are treated with calyculin A, I would expect to see an increase in phosphorylation.These experiments will help to further support the conclusions I have made by showing that the levels of phosphorylation in the spindle either decrease or increase.

After determining additional phosphorylation pathways and localizing myosin within the spindle I would then like to determine what proteins myosin is interacting within the cell. At present, it is unclear exactly which spindle structure myosin is interacting with as it can be the spindle formed by microtubules, skeletor or an additional protein. Determining the proteins that myosin interacts with will help to provide a better understanding of how these complex chromosome movements are accomplished. For example, the Skeletor-formed spindle could be depolymerised during normal bivalent oscillations to see if the same results are found when nocadozole was used to depolymerise microtubules.

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