Microtubule Dynamics During Sperm Aster Centration in Fertilized Sea Urchin Cells

Author: Peter J. Tramontozzi

MICROTUBULE DYNAMICS DURING SPERM ASTER CENTRATION IN FERTILIZED SEA URCHIN CELLS

Peter Joseph Tramontozzi

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Peter Joseph Tramontozzi

Advisor: David Burgess, Ph.D.

Centration of the nucleus after fertilization is an essential step for setting-up cell division and proper embryonic development in many proliferating cells such as the sea urchin. The sperm aster must capture the female pronucleus for fusion as well as the nucleus becoming positioned at the center of the cell. Microtubules (MTs) are known to play a role in this centration but the exact mechanism remains unknown. This begins to investigate current models of nuclear centration and the role of various interactions. Three phases of migration were observed as the male aster migrated with support in independent movements of the male and female pronuclei. Dimpling affects present that altered the morphology of the cell were observed when engagement occurred between the male and female pronuclei. It was discovered that this dimpling effect was a result of an interaction between MTs and the cortex, as confirmed by visualization of sheared cells in which only the cortex remained. Stemming from previous and current research in the lab, the role of post-translational modifications (PMTs) in nuclear centration was investigated for the different forces exerted due to various factors. Tyrosinated and detyrosinated populations were observed with and without the presence of parthenolide (PTL), an agent that inhibits detyrosination. PTL was observed to not only prevent the proper migration, but also that it expanded tyrosination of tubulin – which would further disrupt the force vectors created through the PMTs promotion of dyneins and kinesins. The results have lead to a new hypothesis to be furthered in order to gain an in-depth understanding in the mechanism(s) for pronuclear migration.

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ACKNOWLEDGEMENTS

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1.0 INTRODUCTION

During cell proliferation in most eukaryotic cells the nucleus is repositioned to help establish a spindle axis, plane of division, and alignment of genetic material. These processes lead to the ultimate goal of cytokinesis - with separation of the genetic material into two daughter cells. For example, certain somatic cells position have the nucleus positioned off-centered but still in a manner to align the division plane. For *Canenorhabditis elegans* zygotes, the asymmetric positioning of the nucleus allows for the proper first-division to its respective plane. Echinoderm zygotes, *Xenopus laevis, Danio rerio*, and the sea urchin, exhibit a centering of nuclear material to intiate further cellular events in the organism's first stages of development.

In echinoderms, the process of migration to subsequent centration of the nucleus begins once a sperm enters through the membranes surrounding an unfertilized egg and into the cytoplasm. Upon penetration, centrosomes attached at the male pronucleus radiate astral (MTs) from its pronucleus attached to a centrosome, to then meet the female pronucleus between the pronuclei positions and migrate to the center of the cell [1] (***Figure 1***). Migration of the male pronucleus is a MT-dependent process as shown in experiments

involving *Lytechinus pictus*, *X. laevis*, *C. elegans* and mammalian cells, where Nocodazole treatments disrupt the migration and centration processes [1-3].

Figure 1. Sperm aster migration in a fertilized sea urchin embryo. Immediately after fertilization (0 min), the male pronucleus (indicated by the white triangle) migrates to the center of the cell to meet with the female pronucleus. (Figure adapted from [1])

1.1 PREPARING THE DIVIDING CELL

Pronuclear positioning plays a key role in the development of the organism and is one of the first major steps after fertilization for echinoderms. This step is essential for following cellular events, including the mitotic apparatus being properly positioned for a symmetric cell division.

1.1.1 Setting up a Plane of Division

Evidence that the nucleus dictates division was first presented in 1884 by Oscar Hertwig in a landmark experiment where he flattened frog eggs to create a long axis of symmetry. He found that the cleavage furrow initiates perpendicular to the longest cell axis, with the

zygotes nucleus at its center. Further exploration into Hertwig's discovery would later uncover that the mitotic apparatus orientation and subsequent microtubule (MT) contact with the cortex plays a causal role in determining the plane of a cleavage furrow [4, 5].

1.1.2 Cleavage Furrow Determination

Experiments with animal cells have shown that the position of the furrow for cytokinesis is dictated by the positioning of the mitotic spindle [6]. Bringmann, H. & Hyman, A. have shown in *C. elegans* that the mitotic spindle sends two consecutive furrowing signals to specify the positioning of the cleavage furrow [7]. A defined directive is necessary within symmetrically dividing systems, such as sea urchin cells, to produce daughter cells of equal dimensions. This process for determination and stimulation of the actomyosin contractile ring ingression has been further studied with some basic models having been established.

As shown in ***Figure 2***, various components and types of MTs make up the mitotic spindle apparatus at the center of the cell. Two centrosomes are anchors to the aligned chromosomes by kinetochore MTs (KMTs) as well as through the bundling of spindle MTs (SMTs). In addition, astral MTs (AMTs) nucleate towards both the cell poles and the equator. These astral MTs are known to signal to the cell cortex for division in multiple model systems [7-9]. Propositions point towards the model that a signal is sent from the spindles to the plasma membrane, as shown in the alteration of the mitotic spindle apparatus towards one side of the cortex leading to the premature furrowing at the nearest point of spindle-cortex proximity.

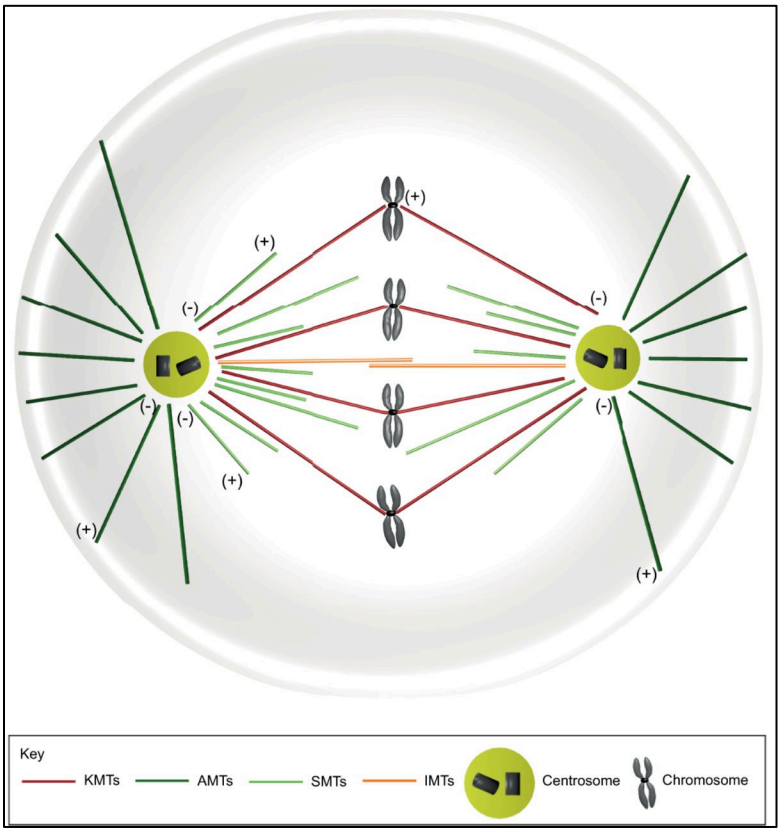

Figure 2: Various groupings of mitotic microtubules. Interpolar MTs (IMTs) connect the two centrosomes while kinetochore MTs (KMTs). Spindle MTs (SMTs) radiate from one centrosome to the other while astral MTs nucleate from the centrosome and towards the cellular cortex [10].

1.2 EXERTION OF FORCES ON THE NUCLEUS

While the importance of a centered nucleus prior to mitotic activities is outline above, it is still unknown how the nucleus finds the center. Previously described in ***Figure 1***, the male pronucleus enters the cell and migrates to the center – resulting in a capture of the female pronucleus [1]. MTs play a role in this centration, but the exact mechanism behind

the migration has not been fully established. Current models of two forces, cortical and cytoplasmic centering, have been offered to explain the method in which the nucleus reaches the center of the cell.

1.2.1 Cortical and Cytoplasmic Centering Forces

There are multiple theories for how interactions between the cell cortex and astral MTs generate nuclear centration forces. First passive forces may be present when astral MTs polymerize against the cell cortex, pushing the aster away from the plasma membrane (PM) and towards the cell center [11]. Expression of this model of nuclear movement is most prominent in smaller cells such as yeast [25-26]. Opposite of the pushing cortical forces resulting from growth are the pulling cortical forces present at cortex. Cortically-bound dynein walks towards the minus ends of astral tubules, which are anchored at the centrosome and connected to the male pronucleus. Most studied in *C. elegans*, the force created from cortically-bound dynein results in a pulling of the nucleus forward towards the PM [12-14]. Park and Rose proposed a model showing a greater concentration of cytoplasmic dynein regulators at the anterior cortex, leading to a greater net pulling force toward the anterior [27]. Under these pulling forces, nuclear centration will occur due to an imbalance between the cortical forces – a resultant from asymmetry of motors at the cortex as shown in *C. elegans*. One model has used RNAi to disrupt cytoplasmic dynein in *C. elegans* and show its negative affect on microtubule organizing center (MTOC) positioning [15].

Cytoplasmic centering forces are driven by transport of internal membrane along MTs by molecular motors [16]. Dynein carrying cargo walks towards the minus end of MTs, which generates a net pulling force on the centrosomes in the opposite direction of its movement [17]. As the male pronucleus enters from outside to the periphery of the cell, an asymmetry initially present in MT length creates a force that equilibrates as the nucleus centers. In addition to dynein, kinesins walking to the plus end of MTs, have been shown to play a role in the organization of the endoplasmic reticulum in mammalian cells and transport of secretory vesicles [18]. Models have shown the male pronucleus following the longest MT within sand dollar embryos in order to center itself in the cell [3]. For sea urchin eggs, the cytoplasmic dynein-mediated forces have shown involvement in male pronuclear migration [1]. Treatment with dynein inhibitor Cileobrevin D results in similar data to that show in Nocodazole treatment. Laser ablation of MTs also resulted in movement away from the ablation site, suggesting that the cytoplasmic dynein was pulling the aster away from the laser [1]. Overall, experimental and mathematical researches of these motors have made clear that they are necessary during pronuclear migration. However, the regulation and exact contribution for each motor type during pronuclear centration is still unclear.

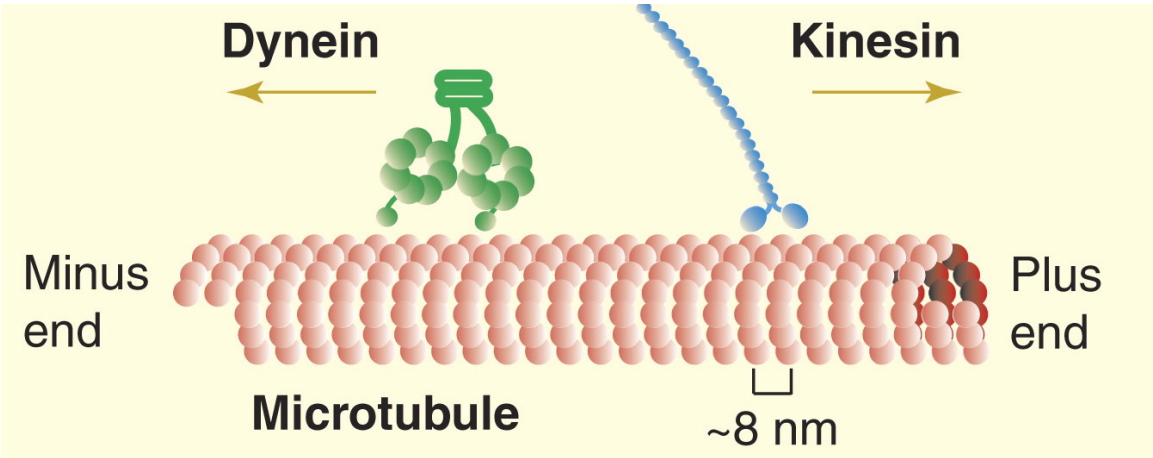

Figure 3: Motors present on microtubules. Motors dynein and kinesin carry cargo and walk towards the minus and plus ends, respectively. A subsequent force is generated in the opposite direction of movement for each motor (Figure adapted from [19]).

1.2.2 Post-Translational Modifications Promoting Various Motors

Post-translational modifications (PTMs) of tubulin have been shown to regulate interactions between MT-binding proteins. More specifically, tyrosination of C-terminal alpha tubulin promotes activity of a dynein protein while detyrosination promotes that of kinesin activity [20-22]. Seen prior in ***Figure 3***, these different motors walk in different directions – thus creating two different net forces. Barisic et al. have shown the importance of MT detyrosination in the guidance of chromosomes during mitosis [23]. Likewise, Barbosa et al. have shown the possibility of dynactin binding to MT with a tyrosination PTM to enhance the dynein-mediated transport and its involvement in the centration of centrosomes [24]. The combination of specifically how kinesin and dynein play a role in pronuclear migration has yet to be shown in sea urchin cells and other echinoderms.

2.0 METHODS

2.1 SEA URCHINS, GAMETE COLLECTION AND CORTICAL ISOLATION

Lytechinus pictus were purchased from Marinus Scientific (Long Beach, Ca), and *Lytechinus variegatus* were purchased from Pelagic Corp. (Sugarloaf, Fl). Both species were cultured in a termperature-regulated aquarium filled with artificial seawater (ASW) made from Instant Ocean mixed at 30-35 ppm. Gametes were collected the day of use by intracoelomic injection of .5M KCl. Sperm could be stored and used for up to ~5 days post-collection. Eggs were collected directly into filtered seawater from the Woods Hole Marine Biological Laboratories. For fixed imaging, eggs were fertilized and passed through a Nitex membrane (86-102 um) several times to remove fertilization envelopes and raised in filtered sea water at the appropriate temperatures until our time points of interest. Zygote cortical isolation was adapted from Henson et al. [28]. Eggs were de-jellied by incubation in acidic seawater (pH 4.0) for 3 min before transferring them back to filtered seawater. We then induced polyspermy by fertilizing the eggs in seawater containing 4 mg/ml of soybean trypsin inhibitor (SBTI). At appropriate time points zygotes were settled onto protamine sulfate-coated coverslips and sheared by pipetting an isotonic buffer containing .8 M mannitol, 5mM MgCl2, 10 mM EGTA, and 10 mM

HEPES (pH 7.4). Coverslips were then submerged in 2% formaldehyde/isotonic buffer for 5 min and processed for immunofluorescence microscopy.

2.2 IMMUNOSTAINING AND STRUCTURED ILLUMINATION MICROSCOPY (SIM)

Isolated cortices were rinsed 3X in PBS/.1%PBT (PBST) over 30 minutes and blocked in 5% BSA/PBST for 2 hours at room temperature. We added Alexa-488 conjugated DM1A alpha tubulin antibody (Sigma) to the samples at 1:1000 overnight at 4C, rinsed 3X in PBST and mounted with 90% glycerol. Images were taken using a Zeiss 880 laser point scanning confocal microscopy with a 40x 1.1 NA water immersion objective. Whole-cell zygotes for SIM and centrifugation experiments were fixed and permeabilized in bulk at the indicated time points using a fixation buffer composed of 100 mM Hepes (pH 7.0), 50 mM EGTA (pH 7.0), 10 mM $MgSO_4$, 400 mM dextrose, 2% formaldehyde, and .2% Triton-X. We excluded 10% diH_2O from the recipe to fill with zygotes in seawater. Samples were incubated for 1 hr at room temperature with gentle agitation, rinsed 3X with (PBST) and left to sit overnight in PBST. We blocked samples in 5% BSA/PBST for 2 hrs at room temperature on a rocker. Samples were then transferred to Alexa-488 conjugated DM1A alpha tubulin antibody (1:1000) in 5% BSA/PBST for 48 hrs at room temperature on a rocker. We rinsed the samples 3X in PBST over the course of 2 hours and added Alexa 647-conjucated phalloidin (Molecular Probes) at 8U/ml during the second rinse. Samples were mounted in either Prolong Gold, Vectashield, or 90% glycerol on chamber slides made from double-sided tape. Imaging was performed using a

Zeiss 880 equipped with an Elyra system. Z-stacks of ~40 um were acquired at .2 um/plane using a 60x 1.4 NA oil immersion objective and processed for SIM with Zen Blue software.

2.3 CENTRIFUGATION AND LIVE CELL IMAGING

Eggs in filtered seawater were distributed over a 1.1M sucrose pad and centrifuged at 10,000g for 6 minutes using a Sorval HB-4 rotor. The eggs were then collected, rinsed 2X in filtered seawater, and labeled for DNA using 1ug/ml of Hoescht. We fertilized the eggs in glass bottom petri dishes and looked for zygotes in which the sperm entered directly opposite of the female pronucleus. We acquired 20-40 um z-stacks every 5 seconds until centration and pronuclear fusion was complete. For data acquisition, we used a Leica SP5 laser point scanning confocal microscope with a 40x 1.1 NA water immersion objective.

2.4 PHARMACOLOGICAL INHIBITORS

For all experiments, inhibitors were added to unfertilized eggs in filtered seawater and pre-incubated for 5-10 minutes before fertilization. Urethane (Sigma, >99% purity) was diluted in DMSO, and used at a 100 uM final concentration. Parthelnolide (PTL) was purchased from Sigma, diluted in DMSO, and used at 50 uM. Control samples were treated with equal amounts of DMSO to experimental conditions.

3.0 RESULTS

3.1 CORTICAL ANCHORING OF MALE ASTER

When approaching the details of the centration of the male pronucleus in a fertilized sea urchin cell, there are several assumptions made. Assumptions that create the basis for a MT length-dependent cytoplasmic pulling model include: requirement of dynein, dictation of directionality through the longest MT and determinants of the movement rate. In addition to testing these assumptions, the basic notion that the aster must be free-floating and not anchored to the cortex was investigated to gain insight in the previously shown cytoplasmic pulling model [1].

3.1.1 Male Pronucleus Can Move Independently of the Female Pronucleus

In order to better understand the assumption of a free-floating aster, there needed to be a consistent method in which aster rates could be analyzed in the presence and absence of forces from the female pronucleus. This was accomplished by setting up the eggs in a manner for which the distance between sperm aster and female pronucleus was maximized. Eggs were centrifuged over a sucrose gradient to position the female

pronucleus at one end of the egg. Fertilization from above then allowed the male aster to enter from the opposite side of the egg.

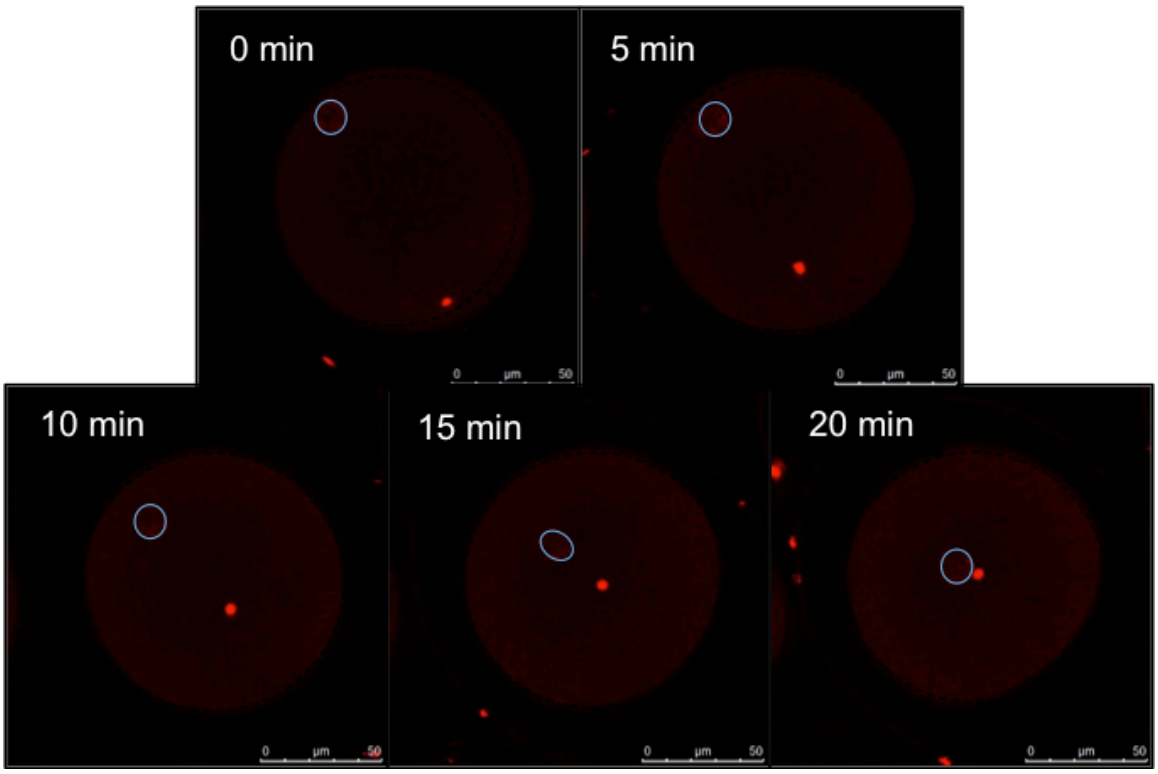

Figure 4: Pronuclei migrations in fertilized centrifuged sea urchin cells. Hoescht-stained cells were spun over a sucrose gradient to cause the female pronucleus (outlined with blue circle) to be located towards the periphery of the cell while the male pronucleus (red dot) entered from the opposite side. 5-minute interval time frames were taken, starting at the beginning of fertilization, to analyze the movements of the pronuclei.

As seen in ***Figure 4***, there exists three phases of movement for the male pronucleus within the 0-25 minute time frame. 0-5 minutes shows the movement of the male pronucleus towards the center with little to no movement of the female, 10-15 minutes show a junction of movement together while 15-20 minutes show the female as the main

moving element. Furthermore, ***Figure 5*** outlines the average distance moved for each respective pronuclei over the time frames indicated. Significant differences between the average distances can be seen before engagement, with the male pronucleus moving, and after engagement, with the female pronucleus moving and as noted by the red outlined box.

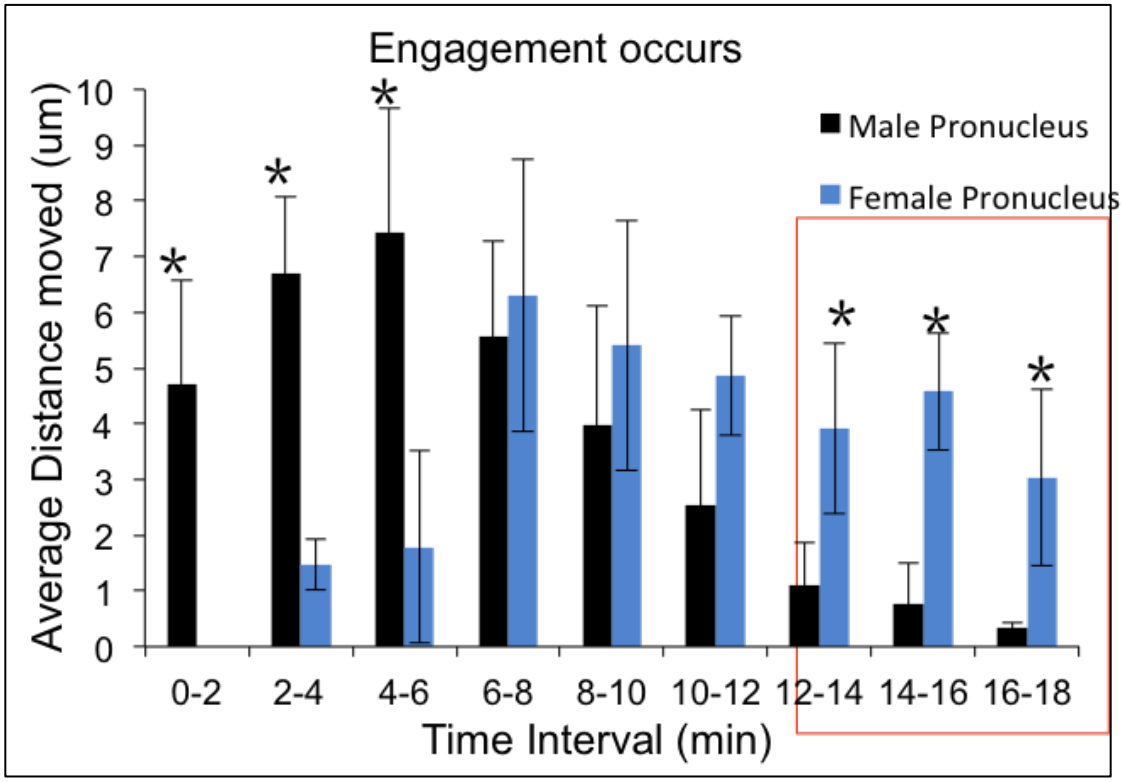

Figure 5: Distance moved of male and female pronuclei during fertilization of sea urchin cells. Male and Female pronuclei were both track during a 20-minute interval post-fertilization to analyze the respective distances traveled in microns. Significant differences between the two distances are noted by a * (n=12) and the interval in which engagement occurs is noted above.

3.1.2 Microtubules Anchor the Male Pronucleus to Rear Cortex

After analyzing the distances travel and movement when maximizing the initial distance between the two pronuclei, evidence indicated the notion of three phases to male pronuclear migration. The first phase exists as the male pronucleus immediately enters the cell and is beginning to center. Shortly after, the next phase is when engagement occurs and the male pronucleus captures the female pronucleus – where the two migrate together towards the center. The final phase exists as the female moves towards the center where the male pronucleus has nearly found, where the two can fuse and begin mitotic events. Looking into the second phase, this engagement and the subsequent forces enacted on the pronuclei provide evidence to the involvement of MTs at the cortex.

Figure 6 shows some of the morphologically effects present as the male pronucleus captures the female. The top section shows the 12-20 minute time frame post-fertilization with a Hoescht stain, showing the migration of the two pronuclei to each other and to the center. However, below are the same images presented on DIC. The 14-minute and 20-minute points provide images of after the male aster capturing the female pronucleus. The rear cortex to the male pronucleus presents a dimpling effect – as seen by the loss of spherically geometry. ***Figure 7*** indicates the percentage of dimpling of zygotes in DMSO in comparison to those incubated and fertilized directly into 100μM of urethane, a drug that promotes MT catastrophe – resulting in decreased MT aster length in a dose-dependent manner [5].

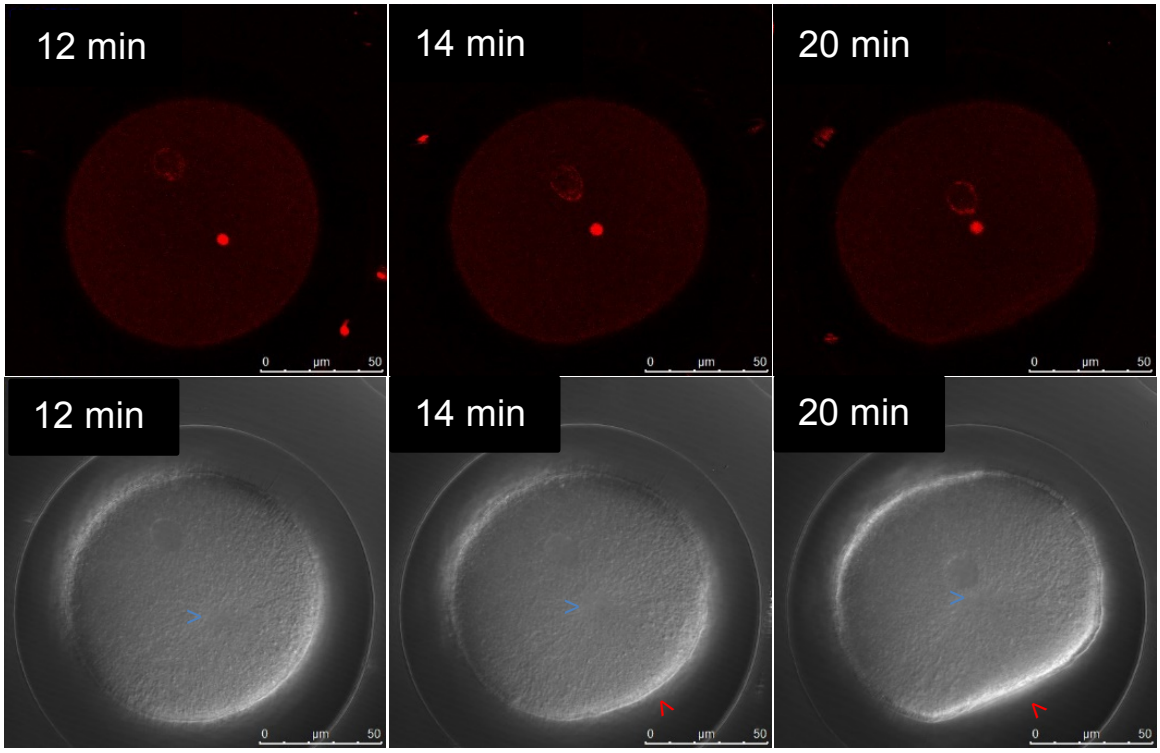

Figure 6: Dimpling of the cortex during male pronucleus migration. 12, 14, and 20-minute time frames are presented post-fertilization. The top-half presents Hoescht-stained cells while the bottom-half shows the same images through DIC. The male pronucleus (indicated by >) can be tracked through the cell, while a dimpling of the cortex is seen (indicated by ^).

Figure 7: Zygotes (n=13) were pre-incubated and directly fertilized into DMSO or 100µM Urethane, where percentage of zygotes expressing dimpling while the male aster migrated was recorded.

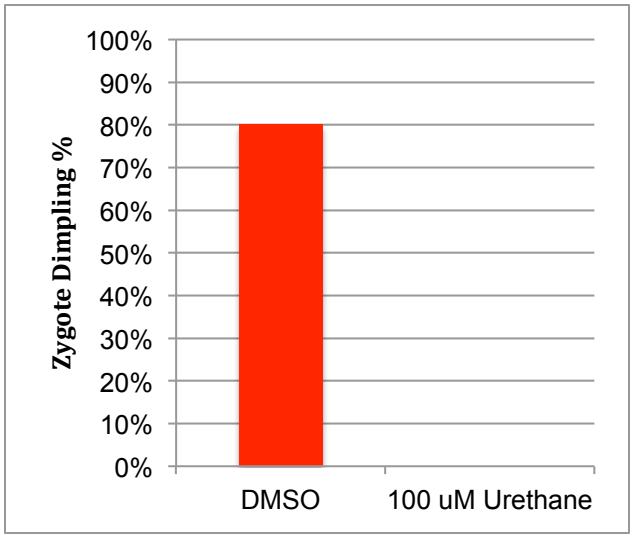

Figure 7: Microtubule dependent dimpling in sea urchin zygotes.

Evidence from the morphological changes during the migration had lead to the postulation of an interaction between the male aster and the rear cortex. To confirm this result, an experiment was conducted to visualize the MTs present. Sea urchin eggs were stuck to a polylysine-coated cover slip and were fertilized in an induced polyspermy manner. These fertilized eggs were then sprayed with an isotonic buffer to shear off the majority of the eggs – while the cell cortex stuck to the coverslip remained intact. ***Figure 8*** shows the results of these sheared cells imaged under immunofluorescence in which MTs were stained. Images show the presence of sperm asters remaining with the cortex that is attached to the coverslip.

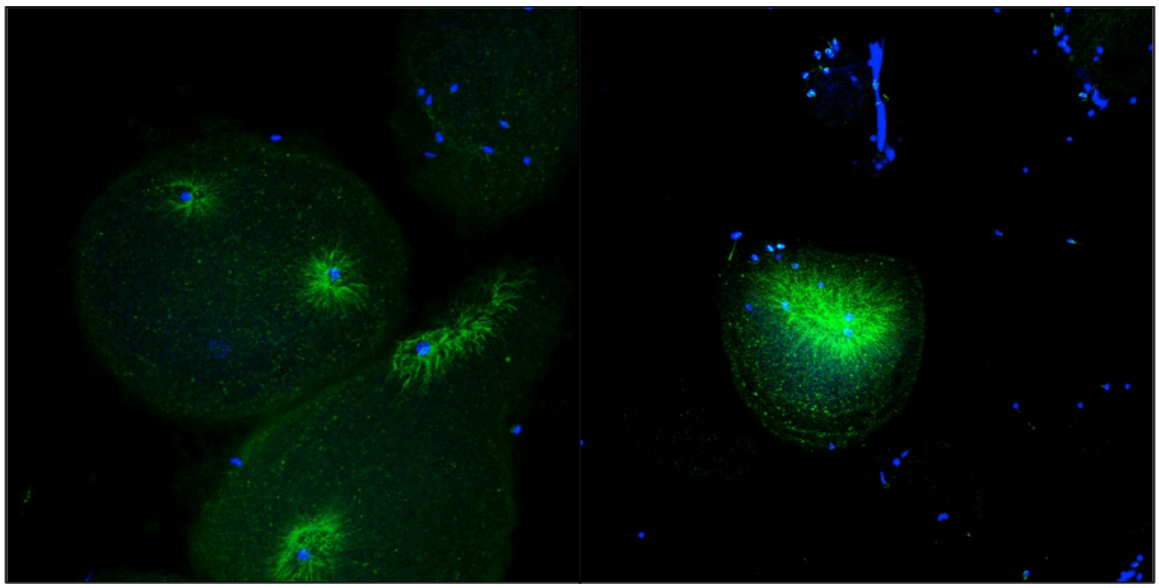

Figure 8: Sheared polyspermy zygotes to visualize cortical elements. Zygotes were stuck to a coverslip and sheared to only leave the cellular cortex remaining. Total tubulin was stained and can be visualized in green while DNA and the nucleus can be seen in blue from a Hoechst stain.

Greater resolution for interactions present between MTs and the cellular cortex was sought after through further microscopy. Zygotes were fixed at a 10 min time point post-fertilization to visualize the male aster as it moves through the cell. Cells were stained with antibodies towards both total tubulin as well as the phalloidin to receive a signal on at the cortex – to then inspect any overlap. The samples were imaged with structured illumination microscopy (SIM) to visualize fluoresced signal. ***Figure 10*** shows the MTs radiating, but lacks detection of antibody from the cortex. Speculation has lead to the lack of signal being a cause of the Vectashield mounting media being used for the zygotes. ***Figure 9*** provides data of the same staining procedure but in varying mounting medias. 90% is to be furthered used to in future procedures to improve data and optimize the visualization of both signals.

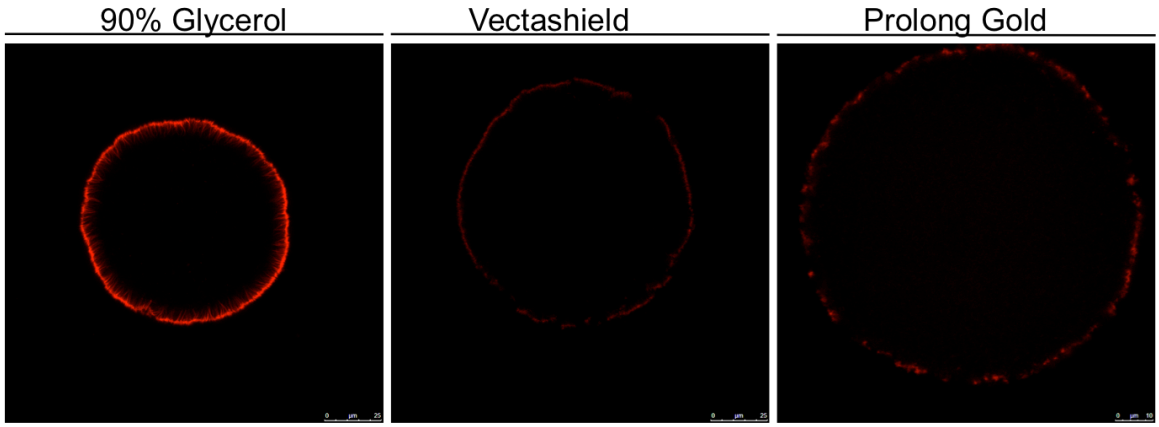

Figure 9: Stains of cellular cortex in various mounting media. Zygotes were fixed and stained with phalloidin to visualize the cortex (red). Various medias were used in order to determine which provided the most signal in order to optimize images for future experiments.

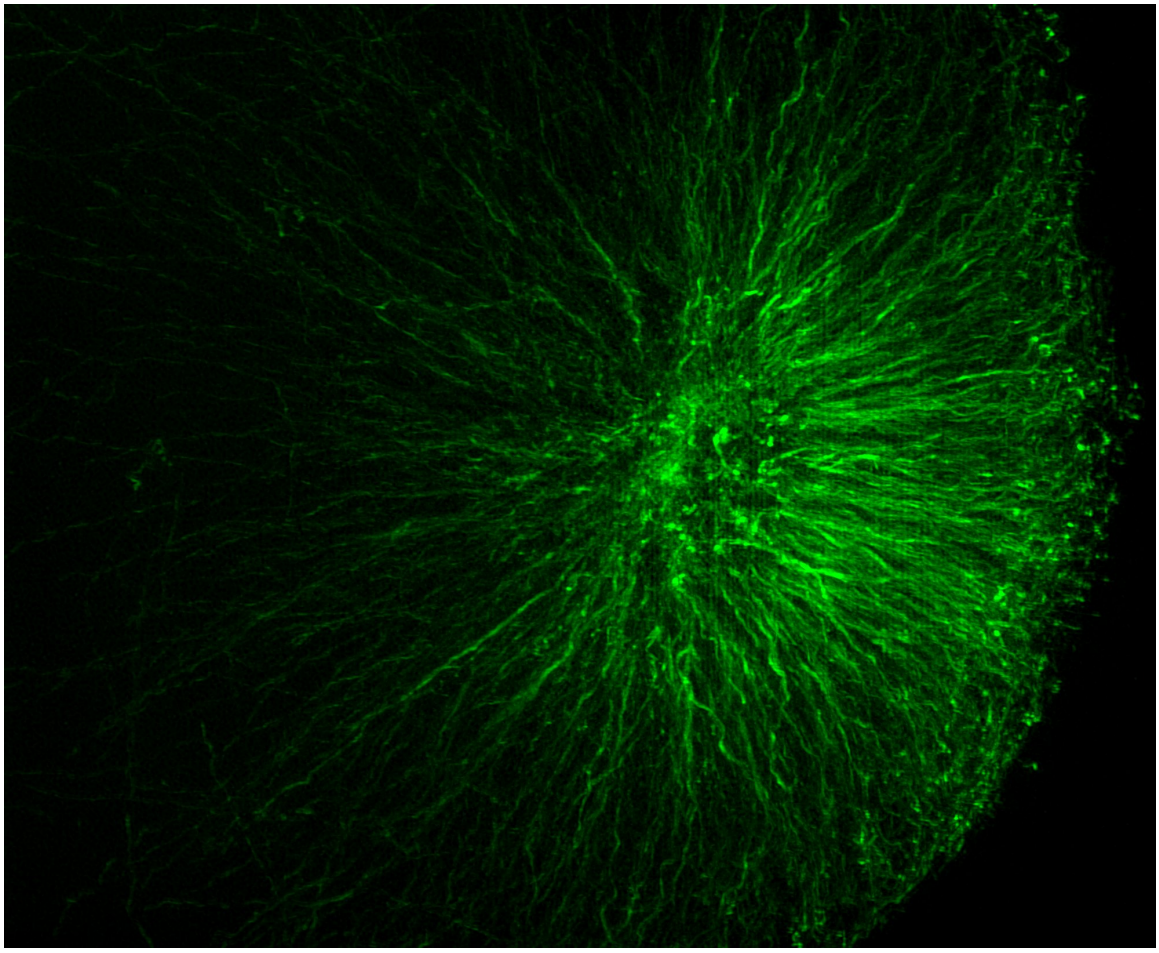

Figure 10: Visualization of sperm aster MTs through structured illumination microscopy. Zygotes were fixed and mounted with Vectashield media to visualize stained microtubules (green) and the cellular cortex stained with phalloidin (red, not shown). Phalloidin signal suspected to be blocked due to Vectashield mounting media.

3.2 MICROTUBULE TYROSINATION-BASED MECHANISM FOR ASTER POSITIONING

Experiments within have lead towards a notion that contradicts some of the basic assumptions presented and required in a cytoplasmic pulling model [1]. Taking a different approach lead to the consideration of post-translational modifications and their roles in the process of male pronuclear migration. Specifically, the role of tyrosination and detyrosination in the recruitment of different motors – with kinesin-1 and kinesin-2 binding preferentially to detyrosinated MTs while tyrosinated MTs being required for dynein motility [20-22].

3.2.1 Microtubule Detyrosination Inhibition Affects Aster Migration

The ability for tyrosination and detyrosination to affect the process of nuclear centering was considered due to previous research conducted. Barisic et al. studied the mitotic apparatus and the alignment of chromosomes during metaphase of the cell cycle in subsequent steps. Using the drug parthenolide (PTL), they noticed detyrosinated MT populations were seen to decrease in its presence [23]. For the story of these PMTs role in nuclear centering in sea urchin cells, PTL was used versus a control to study how the aster migrates. Zygotes were pre-incubated and fertilized into 50μM PTL and imaged under a Hoescht-stain to follow the migration of the male pronucleus versus a control sample. ***Figure 11*** shows the images collected at various time points post-fertilization for those cells in PTL or those not. Control cells exhibited the expected scenario of the

migration of the male aster to the center to join the female pronucleus. Cells with PTL present showed no migration of the male pronucleus to the center fuse with the female.

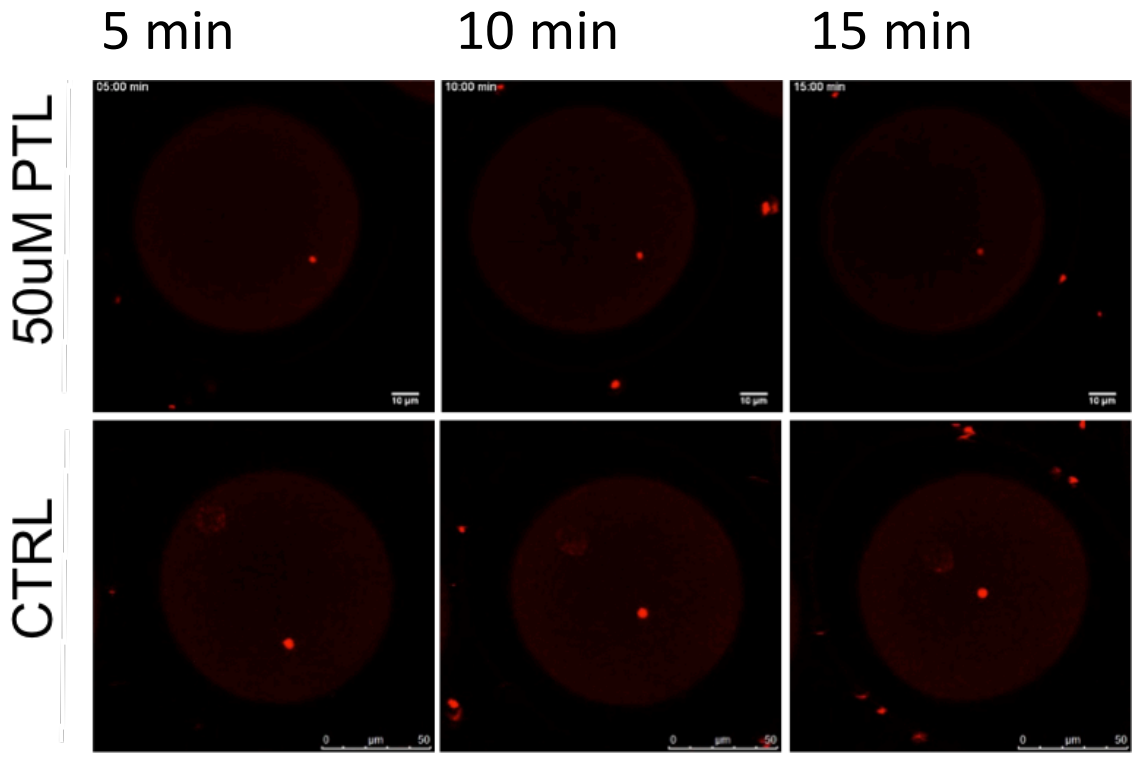

Figure 11: Effects of inhibiting detyrosination of microtubules on aster migration. Cells were fertilized in the presence 50μM PTL to compare to control cells with no pharmaceutical agent added. Cells were stained with Hoescht and imaged under immunofluorescence to track the migration of the male pronucleus, as seen as the red dot in the bottom-right quadrant of the cells.

3.2.2 Tyrosinated Tubulin Expanded through Inhibition of Detyrosination

A greater understanding was sought after for the role of inhibiting detyrosination on MTs and its role in the migration. We used the drug PTL to inhibit detyrosination; another

experiment was set up to compare the inhibited versus the control. Immunofluorescence was used to locate tyrosinated tubulin after the cells were fixed at varying time points. Data shown in ***Figure 12*** shows the effects that an inhibition of detyrosination has on the size of the tyrosinated aster MTs. The images of control data show a smaller size and visualization of tyrosinated tubulin present in the aster. This expansion also coincides with the previous data from ***Figure 11***, as non-fixed cells visualized at further time points still lack the full ability to migrate to center properly and exhibit proper mitotic events.

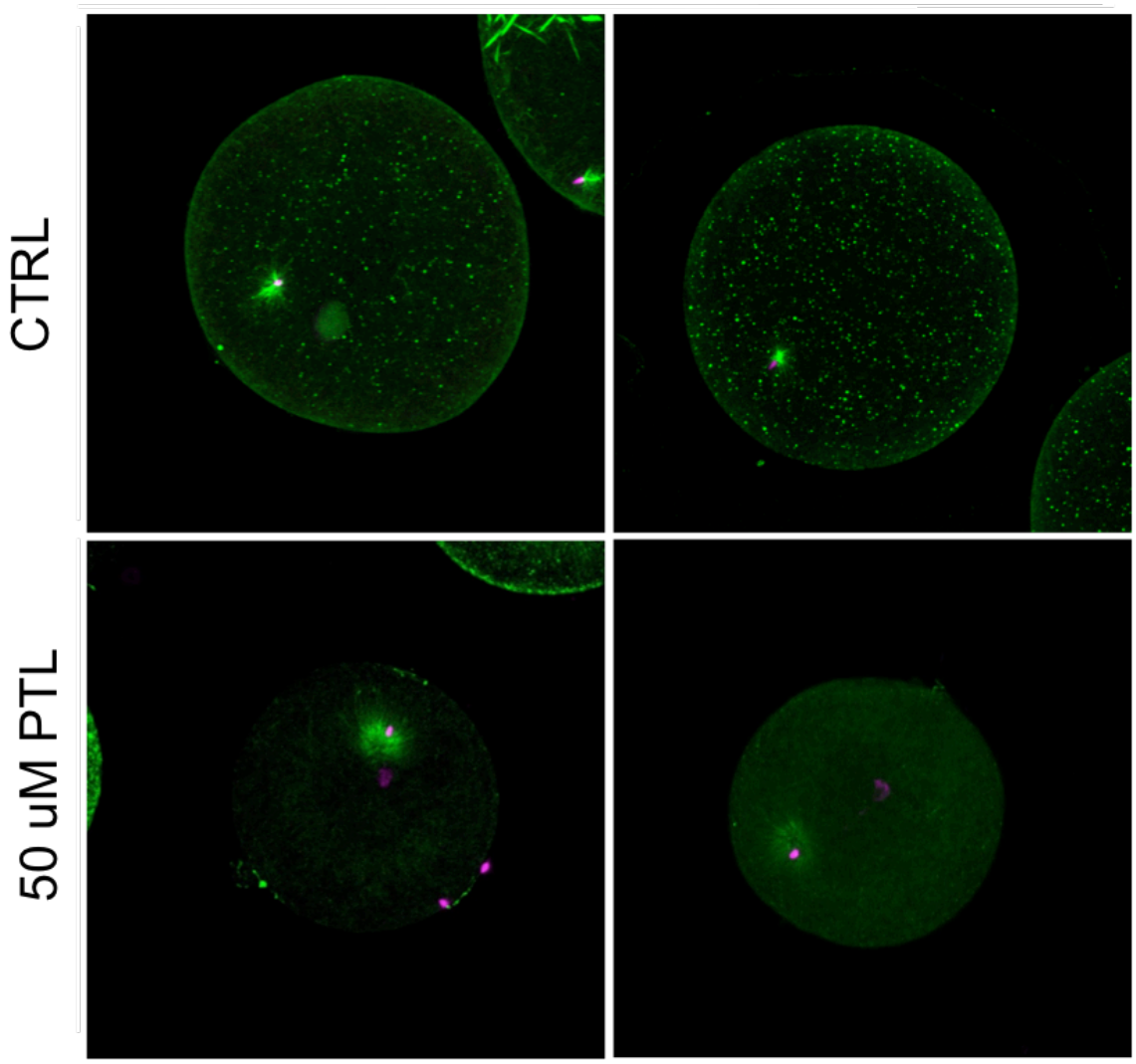

Figure 12: Effects on tyrosinated tubulin size from the inhibition of detyrosination. Control zygotes are compared with zygotes in the presence of 50 μM PTL when visualizing aster size. Zygotes were fixed at a 10-minute time point to visualize the male aster during its migration. Cells were stained with an antibody to detect tyrosinated MTs (green), as well as Hoescht to visualize nuclei (pink).

4.0 DISCUSSION

With a variety of organelles composing a cell, the nucleus is one of the largest and is vital in sustaining the genetic material of a cell. Events occurring originating from the nucleus are essential for mitosis and the overall development of the organism. For echinoderms, losing the centered position of the sperm aster during fertilization could be detrimental to the life of the cell. The exact process and processes of pronuclear migration in multiple models including the sea urchin have yet to be fully established. Evidence has worked to validate some of the incongruities in previous models while also leading to process of establishing a working hypothesis and future mechanism to continue research on.

When the distance between the female and male pronuclei was maximized through a sucrose gradient, three categories of movement were observed. The beginning and end phases focused mainly on the male and female pronuclei, respectively, travelling the greater distance through the cytoplasm. These significantly different distances travelled between the two verified the notion of the sperm aster moving independently of the female pronucleus. The possibility of independent movement led to the likelihood of a different interaction leading to the end outcome of a centration. One way in which the male sperm aster could create its force is through interacting with the cellular cortex.

Morphological changes of the zygote provided evidence of an interaction of MTs at the cortex. This middle phase of aster migration results in the initiation of engagement for the aster capturing the female pronucleus. Models in previous research have shown of this interaction between the aster and female pronucleus has proposed that cytoplasmic dynein-mediated pulling forces lead to the positioning of the nucleus [1]. But this notion requires a fundamental assumption that the male aster is free-floating with a lack of cortical anchoring. Evidence during live-cell imaging showed that when engagement is occurring with forces to have the nuclei and position in the center, a dimpling effect is clearly evident at the rear cortex in respect to the sperm aster. This evidence goes against the assumption that the aster is free-floating. Dimpling was noted to be a result of an interaction occurring somewhere between the male aster and the cortex, with the likely reason resulting from an interaction with MTs.

Confirmation of this interaction was seen through experimenting with fixed-cells and imaging to locate MTs. Cells that sheared to only leave behind the cellular cortex and its interactions showed a positive result for the visualization of sperm astral MTs. This concluded more evidence that the male aster interacts with the cellular cortex through its microtubules. To test this further, experiments were conducted to visualize both total tubulin as well as the actin within the cortex during the male aster migration. Super resolution microscopy is being used to tag both these populations in order to see a possible interaction between MTs and the cortex. Currently, protocols are being modified to optimize images as well as visualize the actin staining – as current data has lacked

visualization from the actin antibody as current mounting media is blocking the fluorophore signal.

Experimental data has supported that the sperm aster interacts with the cortex during its migration towards the center. With the one of the basic assumptions in current models for the migration being contradicted (other assumptions being challenged by Burgess laboratory that has yet-to-be published), there has been a shift in direction towards other possible mechanisms for the migration of the sperm aster. The basis of post-translational modifications that are present and shown in data has been shown within other echinoderms [23-24]. Using this idea has established a working hypothesis, ***Figure 11***, for the forces being exerted to achieve nuclear centration. Two different populations, tyrosinated and detyrosinated MTs, are speculated to create differing force vectors to center the nucleus.

PTL was used to inhibit the detyrosination populations of MTs in cells fertilized cells where the aster was still migrating. The data shows that the male pronucleus was unable to capture the female and properly center in comparison to controls. As explained through the varying populations, a lessening of detyrosination will create a change in the force vector that typically allows for proper migration. The notion was furthered through visualizing that inhibition of detyrosination expanded the size of tyrosinated populations – which would continue to interfere with the proper forces to center the male pronucleus.

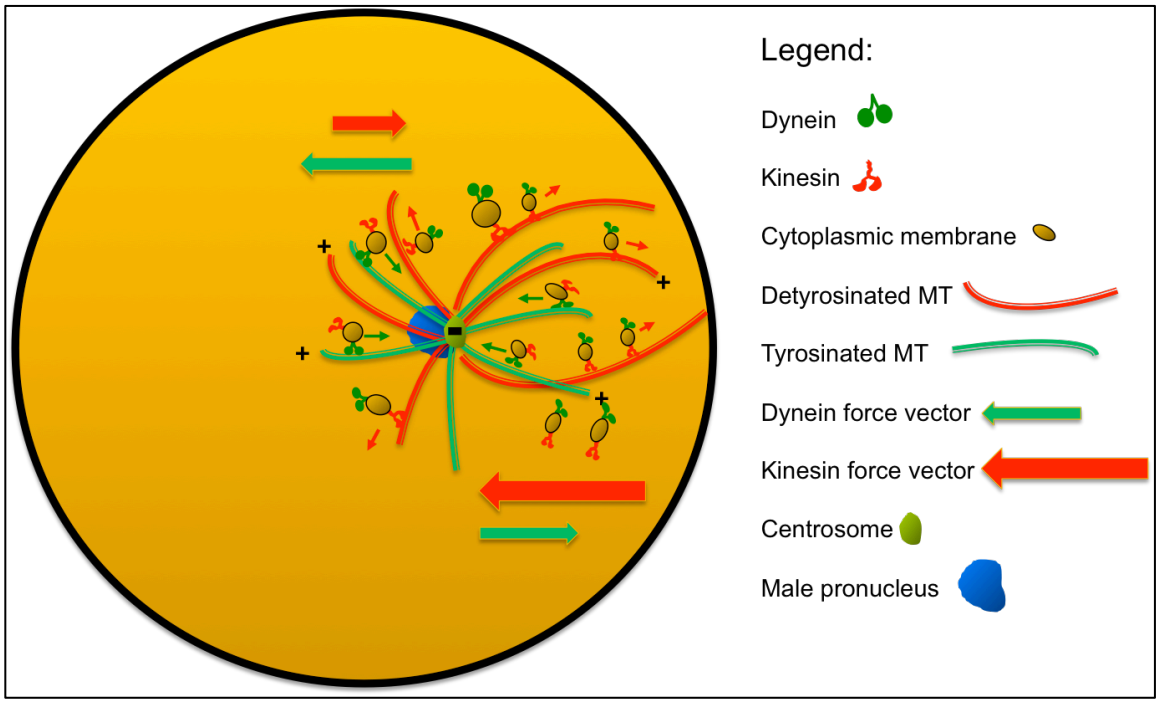

Figure 13: Working hypothesis for movement of male pronucleus. Developed working hypothesis by Johnathan Meaders in the Burgess Laboratory at Boston College. Providing possible mechanism(s) for forces exerted on the male pronucleus as it migrates towards the center to capture and fuse with the female pronucleus.

Experiments moving forward will look continue to understand this hypothesis and the effect of PMTs on the migration. This possible mechanism shows the indication of both pulling and pushing forces being involved to lead to the centration of the male pronucleus. A better understanding of the two populations will increase confidence and support for this hypothesis. Dual labeling of tyrosinated tubulin and total tubulin will provide better insights towards the size of the populations while a Western blot analysis

of tyrosinated tubulin versus total tubulin with and without PTL will confirm the effects of the pharmaceutical agent.

In conclusion, it has been revealed that there exists an interaction between the male aster and the cellular cortex. Fertilizing cells with a maximized distance between male and female pronuclei indicated the notion of independent movement and showed great movements after engagement occur. Investigating the morphology of the cell during this engagement showed a dimpling of the cell, which alluded to the notion of MTs pulling the membrane inward. This was verified by visualizing MTs in sheared cells to which the only the cortex remained. In addition, PMTs were investigated and developed into a working hypothesis for a mechanism in which male pronuclear migration is possible. Different populations of tyrosinated and detyrosinated tubulin were visualized with the drug PTL, to inhibit detyrosination, to investigate the affects and variation of forces with the interference on migration. This research provides evidence in response to current models as well as a footing in a new hypothesis of PMTs to explain the mechanism(s) in the forces for capturing of the female pronucleus and subsequent nuclear centration.

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