An Investigation into Contractile Ring Geometry and Dynamics During Early Divisions In Sea Urchin Embryos

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AN INVESTIVATION INTO CONTRACTILE RING GEOMETRY AND DYNAMICS IN EARLY DIVISIONS IN SEA URCHIN EMBRYOS

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AN INVESTIGATION INTO CONTRACTILE RING GEOMETRY AND DYNAMICS DURING EARLY DIVISIONS IN SEA URCHINS

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ABSTRACT

The contractile ring is pictured, analyzed, and even named under the basic assumption that the geometry of the structure begins and ends circularly and the ring is a single homogenous structure acting uniformly. However, under physiological conditions cell-to-cell adhesions force cells and therefore initial contractile rings into highly irregular and noncircular shapes. To investigate this basic assumption of contractile ring geometry, contractile ring shape of dividing sea urchin embryos was analyzed under three conditions: in seawater where cell-to-cell adhesion is strong, calcium free seawater where cell-to-cell adhesion is minimized, and in microfabricated chambers to artificially manipulate the initial contractile ring shape. We found that contractile ring geometry evolves over time to become circular even when it begins as an irregular shape due to cell-to-cell adhesions or artificial manipulation. By analyzing velocities of specific regions of the contractile ring, it became apparent that there is always a pattern of rounding regions of lowest circularity before overall ring contraction. This pattern suggests that the contractile ring is capable of producing varying forces in a coordinated manner. Therefore the contractile ring can not only be noncircular, but can also possess regions with different molecular and biophysical properties.

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Lastly, thank you to every one of my friends and family who tirelessly helped me along this year and editing my thesis.

INTRODUCTION

The contractile ring is the functional structure by which cells physically divide into two daughter cells to finalize cell division. The contractile ring is responsible for the physical splitting of the cell during cytokinesis. Cytokinesis describes the process of cell division. Thus, a discussion about the contractile ring must first be preceded by a discussion on cytokinesis to put this mechanism into context.

What is cytokinesis?

The contractile ring was discovered to be the cellular component by which cell division proceeds via the process of cytokinesis. In the technical sense, cytokinesis is not a stage of mitosis but is a necessary component for the final division into two daughter cells.[1] Cytokinesis refers to the division of cytoplasm at the conclusion of mitosis as the final event in a series of steps during the process of cell division. [2]. In general, all organisms share the same goal during cytokinesis, to physically separate the mother and daughter cell with as little damage to either cell or cell membrane as possible. While the process of cytokinesis varies slightly among eukaryotes, the major events remain constant. While cytokinesis begins in anaphase, the first visual aspect of cytokinesis is the puckering of the cell surface to form a cleavage furrow. This furrow deepens into the cell until the cell is divided into two, during which the cell membrane wrinkles. Membrane puckering suggested that there was an underlying mechanism responsible for pulling the membrane inward, leading to the discovery of the contractile ring. After the cleavage furrow, cytokinesis continues and can be divided into four distinct factions- initiation, contraction, membrane insertion, and abscission.[3] Even if total volume remains the same between a single cell and two daughter cells, as it is during early divisions of sea urchin embryos, the total surface area of two daughter cells is greater than a single cell. Therefore, for cytokinesis to be successful, significant amounts of new membrane must be added to one or both daughter cells.

How was cytokinesis discovered?

Theories of cytokinesis have been explored since microscopes allowed a closer look at this incredible phenomenon. However, there have been landmark discoveries and highly influential scientists along the way that helped further our knowledge of cytokinesis. One of these scientists is Eduard Strasburger. Strasburger worked to develop cell theory during the 1880s characterizing the discrete stages of cell division as well as the process of cytokinesis.[4] Looking at the coordination of mitosis and cytokinesis, Strasburger studied both uninucleate and multinucleate cells. [5] Looking at a variety of cells, it was Strasburger who was able to conclude that although cytokinesis looked dissimilar it was in fact the same process happening. He not only noted the similarities of cytokinesis but postulated that mitosis and cytokinesis were separate and operated independently, a principle still important to today's understanding of cytokinesis. [6]

What controls cytokinesis?

For cytokinesis to occur without problems, there must be significant interplay between numerous cellular proteins and processes. The central problem for every cell undergoing cytokinesis is to avoid dividing too early in M-phase.[7] If a cell were to divide too early in M-phase, daughter cells may not inherit all the necessary genetic information. Conversely, cytokinesis failure leads to aneuploidy and genomic instability, both of which can be fatal to the cell or may pave the way for future malignancies.[8] Because of the need to prevent premature division and other potentially fatal malignancies, the process of cytokinesis relies on significant interplay between the plasma membrane, underlying cytoskeleton, and signaling molecules associated with the membrane.[9] A myriad of kinases are essential in cytokinetic regulation. Polo kinases, CDKs, an Aurora B kinases all temporally and spatially regulate cytokinesis.[10] The cytoskeleton proteins that regulate cytokinesis include actin and myosin, microtubules, septin, anillin, and ESCRT III. The diversity of the cytoskeleton offers a peek into the many processes that must interplay for cytokinesis to be successful.[11] Cytokinesis, in most cases, is carried out by an actomyosin ring known as the contractile ring that tightens and pulls the membrane to help pinch the cell into two.

What is the contractile ring?

The contractile ring is a structure just below the plasma membrane in dividing cells in many cell types. This ring is composed mainly of actin and myosin but also contains numerous other proteins.[12] This ring structure assembles on the division plane during anaphase and as the nucleus divides, the ring contracts to divide daughter cells.[13] The contractile ring therefore needs to be under tight temporal and spatial control. The ring structure is responsible for squeezing the cell into two, almost like a drawstring purse. It is the overlapping of the actin filaments and bipolar myosin II filaments that can generate the force required to pinch and divide the cytoplasm in two.[14] Overlapping antiparallel actin sliding by myosin II motors generate forces in many different directions, but these forces work in conjunction to constrict the ring as a whole. Myosin II is an ATP dependent motor protein that interacts with filamentous actin. Through actin binding, ATP hydrolysis, and force transduction myosin slides actin filaments. This power stroke action is responsible for muscle contraction as well as contractile ring constriction. However, the uniform thickness of the contractile ring throughout cytokinesis suggest that rather than contracting like the myosin II and actin in muscle cells, there is a slow net loss of actin and myosin II filaments at cytokinesis progresses.[15] The contractile ring is not fully disposed of, however, until cleavage ends and the cleavage furrow pinches inward enough for a midbody to form between the two cells.[16]

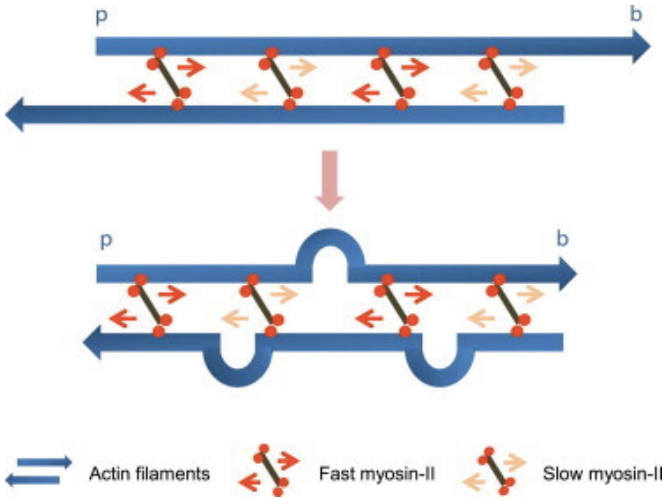


Figure 1. Different myosin velocities promote actin buckling. (adapted from Figure 6 of Lenz et al., 2012)

How was the contractile ring discovered?

Several theories were proposed for centuries about the mechanism that was responsible for the division of cells. It was understood that there must be a physical mechanism for dividing cells, but theories ranged widely. Thomas Schroeder brought light to the field in the 1970s when he proposed the idea of a ring of filaments as a mechanism for the division of cells. [17] By looking at fertilized sea urchin eggs, he noticed that there is a concentration of circumferential filaments that forms after anaphase and exists through cytokinesis. Through electron microscopy, he saw that this structure seemed to decrease in width as the cell divides. He postulated that due to the decreasing volume of this ring, there must be some break down of the ring, which he calls contraction-related disassembly of contractile ring filaments. Since this breakthrough in exploration, there have been significant gains in learning about the contractile ring and the importance of its role in cell division. However, there is still a great deal that remains to be explored. There are many important questions to be answered. How is the contractile ring specified? How exactly is the contractile ring assembled? Finally, how does the contractile ring coordinate constriction? [18]

Do all cells use the contractile ring to divide?

While many animal cells use the contractile ring to divide, some do not and no plants utilize this mechanism.[19] Since plants have a cell wall, they have no need for a contractile ring. In the same sense, there are many cells that do not have a contractile ring and appear to not need it. There are cells that are myosin-depleted but still seem to divide in a fairly normal manner, pointing to alternative methods of completion of cytokinesis. [20] Membrane insertion during cytokinesis can contribute to the varying types of cytokinesis that can go to completion without a contractile ring. This points to the contractile ring being a key piece of the story, but is not the whole story when it comes to cytokinesis.

How does the contractile ring align in the cell?

Proper alignment of the contractile ring is absolutely crucial during cell division. Improperly aligned contractile rings produce two uneven daughter cells. While uneven daughter cells can be the desired outcome, for many cells this can be detrimental as the daughter cell may not get sufficient cytoplasm, proteins, or membrane in the split. The position at which the contractile ring aligns is determined by the positioning of the mitotic spindles. While the mechanism is not completely understood, it is known that the microtubules of the mitotic spindle position the ring at a point equidistant from the two spindle poles. [21] There is a vast array of proteins that have been identified as significant factors in the positioning of the contractile ring in dividing cells. Proteins involved often organize actin in some way and include: actin, myosin, septins, formins, Arp2/3 complex, tropomyosin, coronin, anillin, profilin, IQGAP, filiamin, MLCK, ROCK, ect. [2223] There exist many molecular signals that cue the position of the contractile ring. The localized activation of the small GTPase Rho at the cell equator may contribute to control the position of the contractile ring. Rho regulators that concentrate on spindle microtubules at the cell equator transmit the signal to activate Rho from the microtubules to the cell cortex. GTPase RhoA influences several downstream effectors (like protein kinase ROCK and citron) to promote myosin activation and actin filament assembly at the cortex of the cell. [2425]

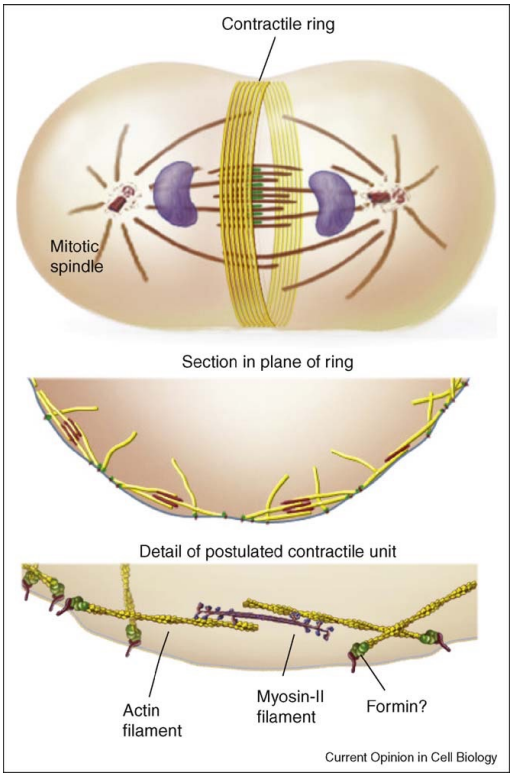


Figure 2. Schematic of the Contractile Ring. Adapted from Current Opinion in Cell Biology (Pollard, 2010).

What components make up the contractile ring?

There are numerous proteins that play a vital role in contractile ring assembly and movement. Actin was first found to be a major component of the contractile ring, with myosin involvement discovered soon thereafter.[26] [27] Specifically, filamentous

actin and myosin II are utilized. More than 50% of actin in the cell is in the form of actin monomers, so the actin needs to be concentrated to the cortex of the cell as filamentous actin.[28] F actin is polymerized from profilin bound actin monomers with the help of formins forming a relatively stable bundle of F-actin important in creating the force required for cytokinesis [29]. Additionally, since actin must be dynamic to prevent contractile ring arrest during cytokinesis, cofilin, a severing protein, is utilized.[30] The tag team of proteins allows for a stable F-actin to produce force when needed while severing that same filament once its role has been served.

The contractile ring itself is mainly an acto-myosin structure, however for cytokinesis to be successful it must be tethered to the plasma membrane. This is where anillin plays an important role by aiding in the attachment of the contractile ring to the plasma membrane. Anillin binds directly to the spindle associated component RacGAP50C in addition to actin and myosin within the contractile ring. [31]Both of these associations are needed in order for the contractile ring to stay coupled to the equatorial cortex, so anillin is vital in both the positioning and the maintenance of the contractile ring. Without anillin securing the interaction between microtubules and the contractile ring, cytokinesis fails.[32]

By what mechanism does constriction occur?

Interestingly the contractile ring seems to maintain a uniform thickness throughout constriction. EM sections of the contractile ring show that the width of the ring stays relatively the same throughout contraction. For this to be possible, overall levels of proteins involved in the ring must be decreased over time. This suggests an overall decrease in involved protein to maintain a constant thickness and avoid contractile ring auto arrest. [33] If no depolymerization of actin and disassembly of the ring occurred, contraction would arrest because the ring gets too thick and constriction can no longer occur. The ring is able to constrict through polymerization and disassembly, forming a type of purse string action.[34] Through this purse string motion along with polymerization and disassembly, force is generated while maintaining a uniform thickness.

There are several specific models that attempt to account for the relatively slow but dynamic constriction; however, none are clear in accounting for every aspect of ring movement. Most models agree that force is produced in part by a randomly distributed network of actin in many different directions.[35] It is through this mechanism of action that actin and myosin II are able to produce a force capable of causing a circular constriction. Therefore while similar players are involved in muscle contraction, the unique organization allows for a multidirectional force that combines to form a contracting motion.

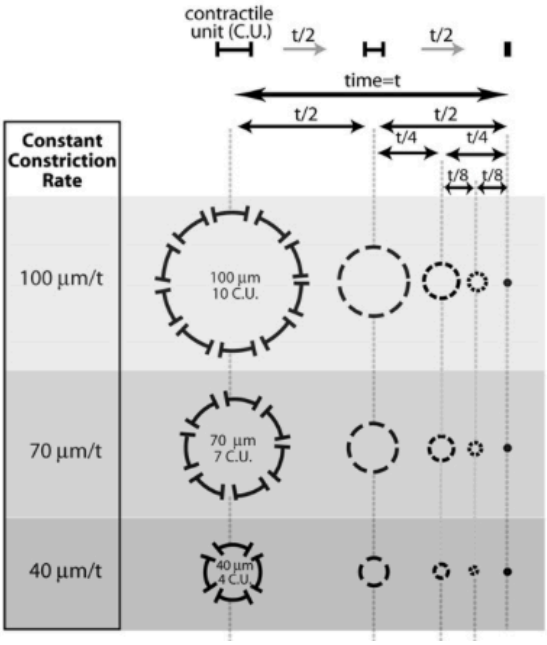


Figure 3. Schematic describing the contractile units model of the contractile ring. (Carvalho et al., 2009)

It has been shown that constriction occurs in two phases, a first phase in which constriction is constant and a second phase during which constriction is proportionate to the perimeter of the ring. [36] Through this model, uniformly assembled "contractile units" account for this variability in constriction rate, with larger contractile rings containing more of these identical units. Contractile units may be assembled individually, then as the ring contracts; whole units are lost to dissemble parts of the contractile ring that are no longer needed. No model currently explains the disintegration of the contractile ring at a certain point during cytokinesis.

The contractile ring has captivated researchers' attention for decades, and continues to do so. On the surface it seems to be an example of a problem with a rather straightforward answer; a contracting force pinches a cell into two, which solves the

basic problem of cell division. However, upon closer inspection the phenomenon is much more complex than it appears. It is these complexities that remain elusive to this day. Studies into the evolving shape of the contractile ring remain lacking. Most insights into the dynamics and mechanisms of the contractile ring are made under the assumption the contractile ring begins and ends as a circular structure. However, upon closer inspection, it's clear that the ring does not always start as a circle. As cytokinesis progresses, the noncircular shape evolves to a circular structure. This indicated that portions of the contractile ring must be moving faster than others. This phenomenon was maintained under natural contractile ring constraints utilizing cell-to-cell adhesions and artificial ring constriction through the use of microfabricated chambers. [37] Its importance on every level, from cellular to organismal, cannot be overstated, so the contractile ring remains a fascinating and important area of research.

Contractile ring shape and velocity were measured in embryos raised in seawater, embryos raised in calcium free seawater, and embryos made rectangular by PDMS microfabricated chambers. The contractile rings in embryos raised in seawater were forced into highly irregular, noncircular shapes by the presence of cell-to-cell adhesions. The ring began in this irregular shape before rounding to become circular, so velocities were measured for different regions of the contractile ring to examine dynamics of the shape change. Embryos were then placed into PDMS microfabricated chambers to artificially manipulate the contractile ring into a rectangular shape, and it was found again that the ring rounds to become circular. Velocities were measured for specific regions to determine dynamics. Rounding suggests an uneven production of force, so actin distribution was analyzed to determine whether uneven force was the result of an uneven distribution of actin. Overall, evidence suggests that the contractile ring geometry plays a significant role in initial velocities of certain regions of the contractile ring. Geometry dictates that rounding occurs before overall constriction. This difference in biophysical behavior throughout the contractile ring implies that the contractile ring is not a homogeneous structure

MATERIALS AND METHODS

Methodology Behind the Use of Sea Urchins

Sea urchins have long been used as a model organism to examine problems in cell biology. The eggs collected from sea urchins are relatively large and malleable. Additionally, the rapid and synchronous divisions allow for visualization of cytokinesis and development. Species used were *Strongylocentrotus purpuratus, Lytechinus variegates,* and *Lytechinus pictus*.

Sea Urchin Embryo Culture

Methods to collect gametes, fertilize eggs, and raise embryos were as previously described.[38] Sperm and egg were collected by injecting either *Strongylocentrotus purpuratus, Lytechinus variegates,* or *Lytechinus pictus* with .5 M KCl in order to induce shedding. Sperm was collected dry, while eggs were collected by placing female urchins upside down over a beaker of seawater. Once eggs were collected, the jelly coat was removed by mixing eggs in a beaker and allowing them to settle three times in seawater. Eggs were placed in artificial seawater containing 4 mM p-aminobenzoic acid (PABA). Eggs were fertilized by adding approximately 10 ul dry sperm for every ml of packed eggs. The PABA worked to prevent crosslinking that would harden the fertilization envelope. After centrifugation of fertilized eggs, the water containing sperm was removed, and the eggs were passed through Nytex, before the eggs were suspended in seawater, artificial sea water, or calcium free sea water. While PABA served to chemically strip the fertilization envelope, passing fertilized eggs through a Nytex with a diameter smaller than the egg served to mechanically strip the eggs of the fertilization envelope. *S. purpuratus* eggs were passed through a 74 um Nytex, *L. variegates* was passed through an 83 um Nytex, and *L. pictus* was passed through 98 um Nytex. Embryos were then raised at 12°C, 25°C, and 18°C respectively.

Preventing Di-tyrosine Crosslinking of the Fertilization Envelope using PABA

Di-tyrosine bonds that form to harden the fertilization envelope can be an impediment to working with clear, naked eggs. Therefore, para-aminobenzoate

(PABA) can be a powerful tool in preventing the crosslinking. Consisting of a benzene ring substituted with an amino group and a carboxyl group, PABA is an effective nontoxic inhibitor of ovoperoxidase, which is an enzyme that forms these crosslinking bonds. Without the crosslinking of bonds, PABA in combination with Nytex can strip the egg of the fertilization envelope. PABA performs a sort of chemical stripping while Nytex performs a mechanical stripping by choosing a Nytex with a diameter slightly smaller than that of the egg.

Plasma Membrane Dyes

CellMask and FM4-64 were the two main cell membrane dyes used. CellMask is an amphipathic molecule with a negatively charged hydrophilic dye which aids in anchoring the CellMask probe into the plasma membrane (Life Technologies). FM 4-64 is a lipophilic styryl dye that inserts into the outer leaflet of the plasma membrane (ThermoFisher). FM 4-64 excites at 515 nm and emits at 640 nm. Both dyes follow similar incubation procedures. Eggs were fertilized and stripped as previously described. CellMask, FM4-64, or both were added to create a 1:1000 dilution with concentrated embryos. This mixture was then incubated with shaking in the dark for 15 minutes, before the embryos were washed 3X with seawater or calcium free seawater.

Microinjections

Microinjection needles were made using Kwik-fil borosilicate glass capillaries on a PP-830 micropipette puller. A 10 mg/ml dilution of protamine sulfate to DI water was used to coat coverslips with an overall positive charge, so that embryos would be adhered to the coverslip to aid in microinjection. LifeAct Ruby was purified as described in the Ibidi manual and put into artificial seawater as an injection buffer. Using a Picospritzer II (Parker Instrumentation), ~5% of the total cell volume was injected into a single blastomere of a 2 cell stage embryo. The uninjected blastomere served as a control to ensure that division was unaffected by microinjection of LifeAct.

Microfabricated Wells

The Chang Lab at University of California San Francisco graciously made polydimethylsiloxane (PDMS) chambers.[39] Wells were made from four different molds, each containing variously shaped chambers. The PDMS slab with hundreds of rectangles with various aspect ratios was used in order to manipulate the shape of the embryos. PDMS slabs were activated by placing them well side up in a plasma cleaner (PDC-002; Harrick). A dense aliquot of fertilized eggs was placed on a clean coverslip and the PDMS slab was placed on top. Excess seawater was removed in order to lower the PDMS so fertilized eggs would be forced fully inside the chambers.

Microscopy

All imaging done was live cell imaging. For laser scanning confocal microscopy, images were obtained using a Leica SP5 system.

Data Analysis

ImageJ and MATLAB were both utilized to analyze images collected. Perimeter was measured over time to determine overall rate of retraction of the contractile ring. Eight points around the contractile ring were chosen to measure velocity of specific regions of the contractile ring. Point 1 was the top middle portion of the cell tracing to the center of the contractile ring, and each point was defined by radiating out 45° from the center of the contractile ring going clockwise.

To determine whether or not a difference in data was statistically significant, a p value of p=0.05 or 5% was chosen. Chi squared analysis was used to compare expected and observed values using the equation $x^2 = \Sigma((o-e)^2/e)$. Since the significance level was set at 0.05, a chi square distribution table was used to approximate the p-value.

RESULTS

Analysis of Division in Four Cell Embryos in Seawater

The contractile ring is always thought of as a circular structure, from start to finish. However, cells in an embryo or organism are often constrained on multiple sides by adjacent cells. This is true in the case of sea urchin embryos; especially in the four-cell stage in which each cells is adhered to two other cells through cadherin junctions. To investigate contractile ring dynamics, the division as the embryo goes from four cells to eight cells was analyzed. The third was chosen because the third division plane is perpendicular to the previous two, meaning the division plane would be in line with the coverslip for easier analysis. The third division was Cadherins, specifically CAD-1, provide the main source of adhesion between cells during embryogenesis in sea urchins.[40] Cadherins are a class of calcium dependent adhesion receptors that are responsible for mutual connection between cells. These provide the main counter force for the contractile ring at that division, as the contractile ring needs to pull against the cadherins and release that adhesion to contract.

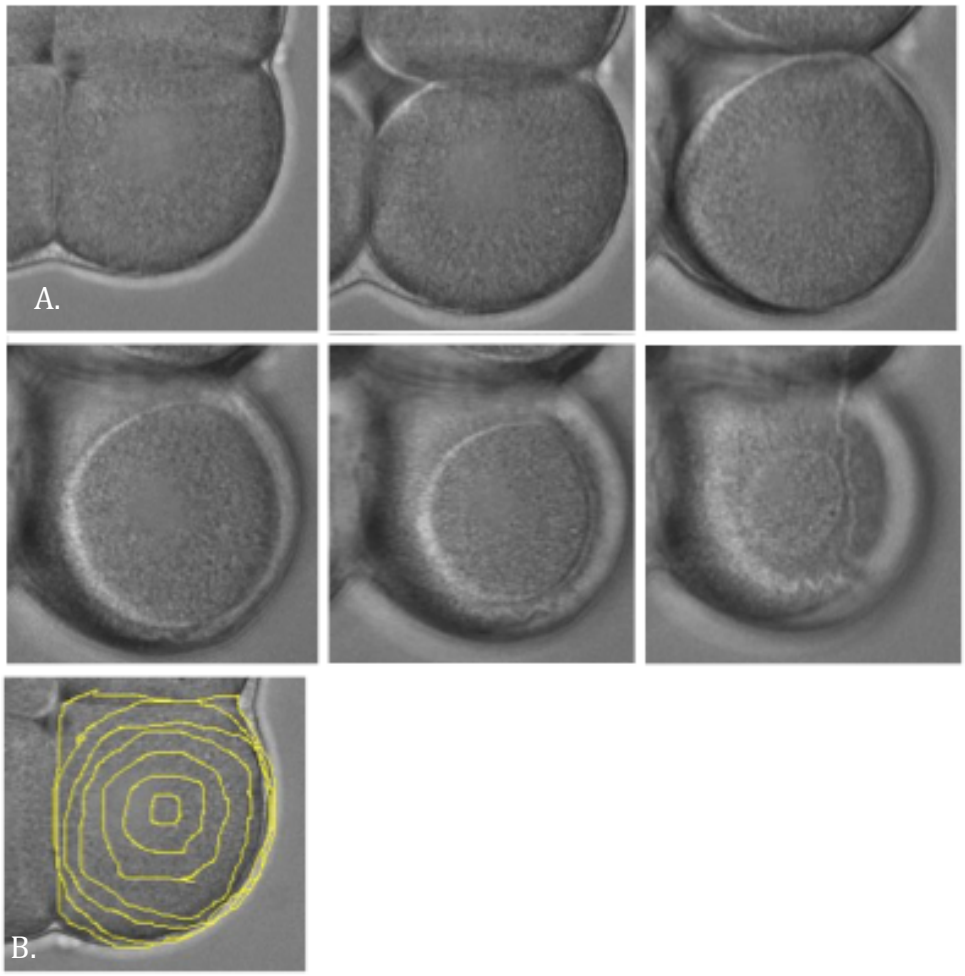


Figure 4. Contractile Ring Shape of *L. Pictus* in SW. (A) Contractile ring shape was analyzed at 20 seconds intervals during the third division using brightfield. (B) Ring shape was traced over time for clarity.

Cell to cell adhesion forces the contractile ring to begin with two relatively straight sides connected by an unconstrained, more circular side (Fig. 4A). This provides a natural manipulation of the starting contractile ring shape. However, over time the contractile ring becomes circular before ending in almost perfect circle (Fig 4B). To go from a noncircular shape to a circle, some regions of the contractile ring must be moving faster than others. Initial measurements were taken to analyze the perimeter of the contractile ring (Fig. 5). Approximately 15 divisions were sampled. This gives an idea of the rate of retraction of the ring. However, the full story is more complicated since regions of the ring must be moving at various speeds. To analyze this, the velocities of eight different points of the ring were analyzed. Points were

chosen by radiating lines from the center of the cell every 45° (Fig. 6). Distance from each points to the center of the contractile ring shows that each point is moving a little differently, as each line has a unique slope (Fig. 7A). Average velocities of each point were calculated over a period of 200 seconds, approximately the time from the beginning of ingression of the contractile ring to conclusion of cytokinesis (Fig. 7B). By tracing velocities of each point, an interesting pattern emerges. For those points where circularity is lowest (4,6,8) the fastest velocities came between the initial time points. Conversely, regions with higher initial circularity to begin with had peak velocities at the second time point. Average velocities throughout cytokinesis are more uniform. This suggests that rounding of the contractile ring occurs before an overall contraction.

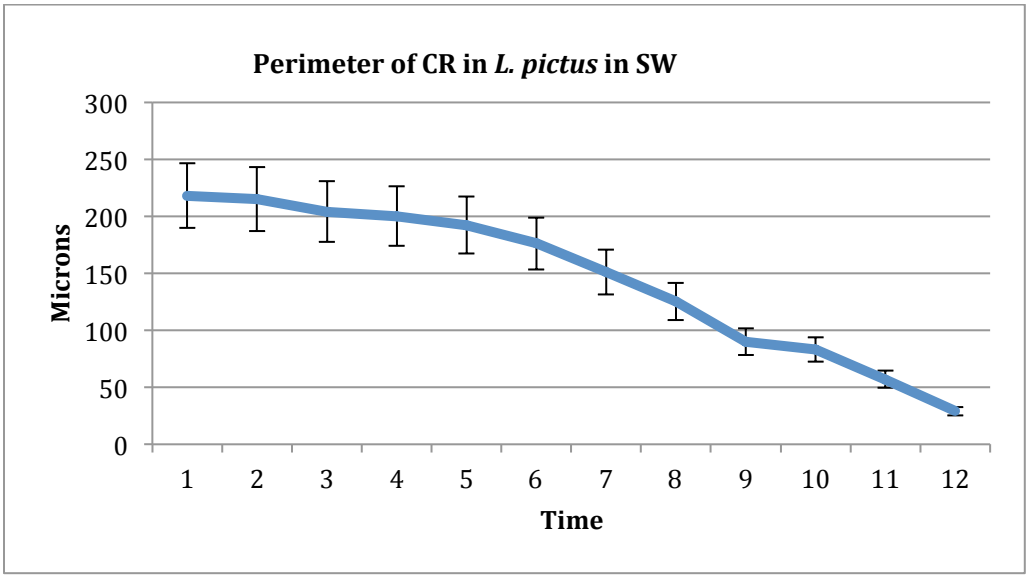


Figure 5. Average perimeter of the contractile ring throughout division of *L. pictus* in seawater. N=15.

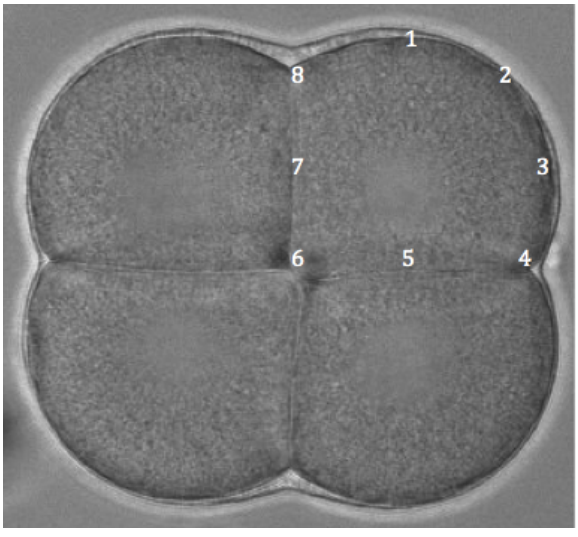
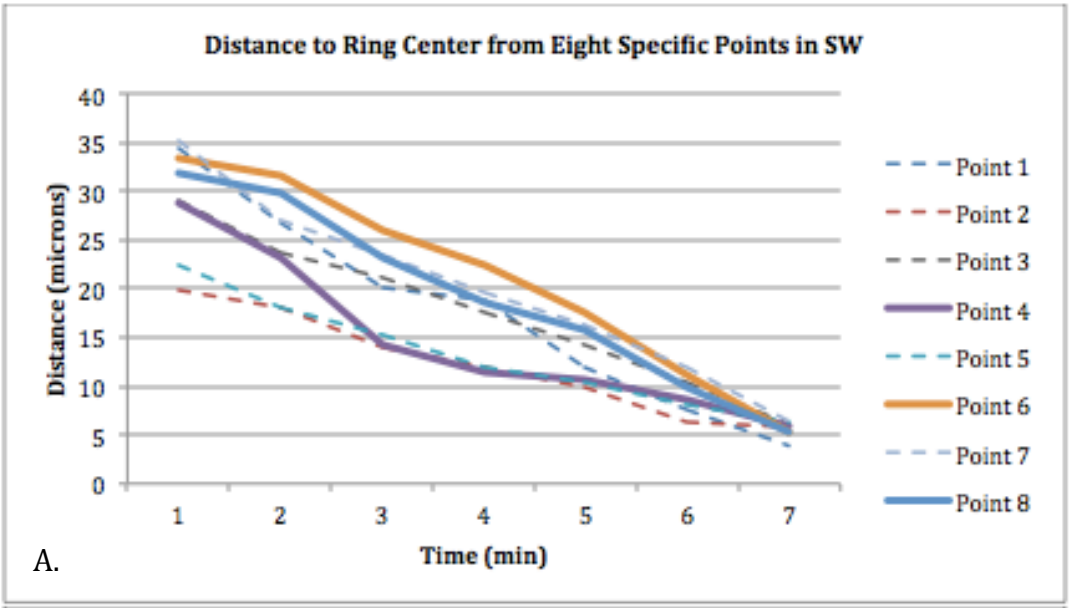


Figure 6. Schematic showing how specific points around the contractile ring were numbered to trace movement. The first point is the center top, while each consecutive point is 45^0 clockwise from the center.



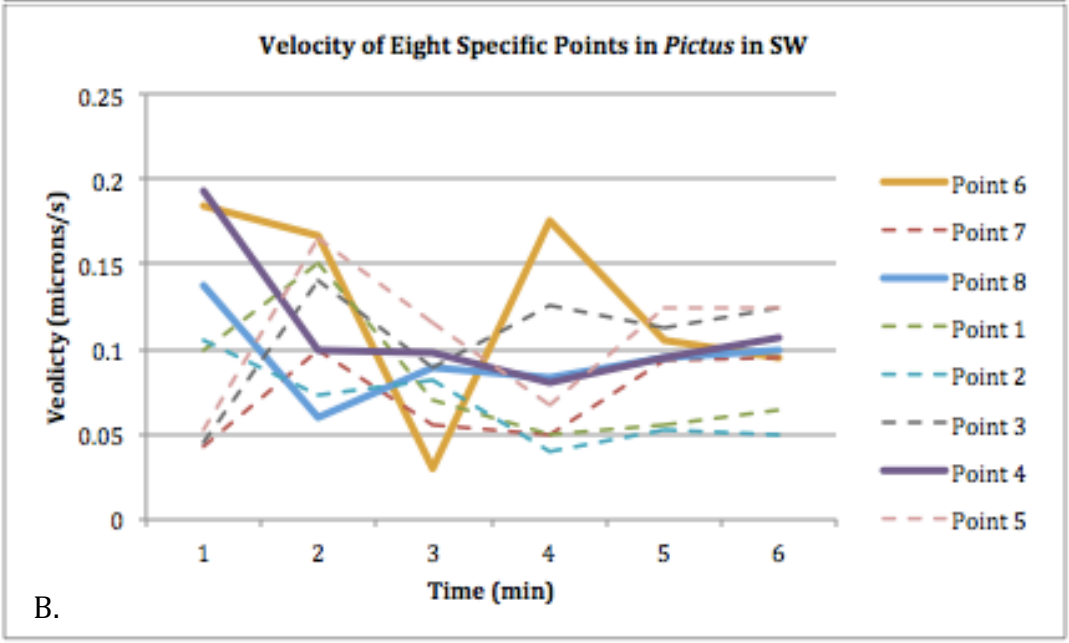


Figure 7. Distance and velocity measurements of contractile ring in seawater. (A) Distance from eight specific points to the center of the final visible contractile ring was measure over time. (B) Velocity was measure for movement between each time point to get a closer look. Points were labeled by naming the top middle of the cell "1" and turning clockwise 45º from the center of the contractile ring to label the next initial point. The specific points on the contractile ring were followed by maintaining the angle in relation to the center point. N=15.

Analysis of Division in Four Cell Embryos in Calcium Free Seawater

Because cadherins provide the majority of the counter force to the contractile ring, they were inactivated to examine the role of that force on contractile ring velocities. Cadherins require calcium in order to function. In the presence of calcium, residues within the cadherins bind to the calcium ions and turn into stiff hooks that link the cells together. Without calcium, cadherin components are linked loosely and the structure is weak, thus cell-to-cell adhesion is lost. This process was utilized to examine contractile ring shape and dynamics without a strong cadherin counter force by raising embryos in calcium free seawater (CaFSW).

There was a clear distinction in morphology between embryos raised in seawater versus embryos raised in calcium free seawater, due to diminished cell-to-cell adhesion (Fig. 8). This gives the initial contractile ring a much more circular shape, and as cytokinesis progresses the contractile ring again becomes highly circular (Fig. 9). The overall perimeter of the contractile ring was again measured over time as conventionally done when looking at contractile ring dynamics (Fig. 10). However, looking at velocity of specific points along the contractile ring (as measured previously), the pattern differs from the velocity of specific points in SW. The points of interest (4,6 and 8) don't have the same pattern of highest initial velocity to account for initial rounding. In SW it appears that drawing in points with the least circularity happens before overall constriction, but in CaFSW, that initial rounding is not necessary (Fig. 10B). Approximately 15 samples were collected.

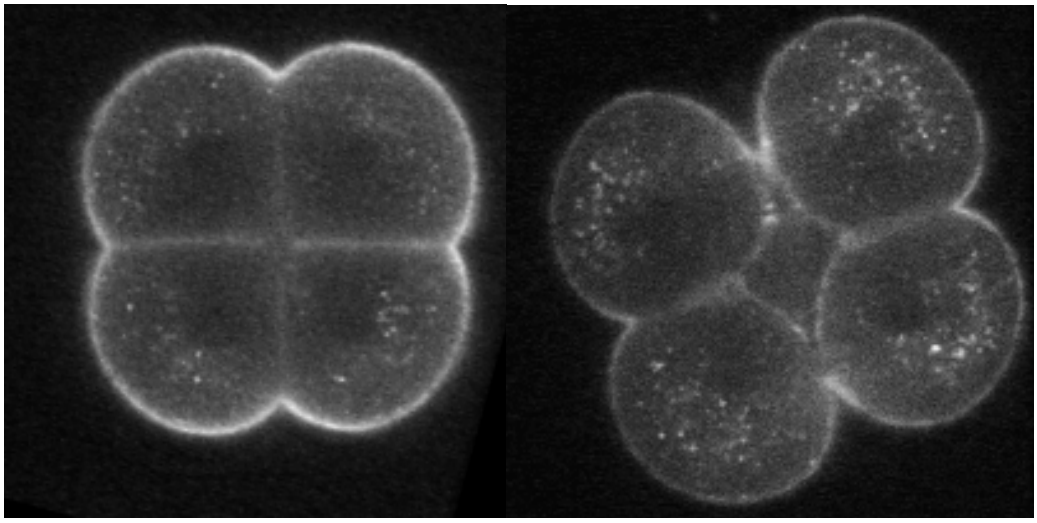


Figure 8. Morphological difference between embryos raised in seawater versus calcium free seawater. (A) Embryo raised in seawater where cadherins are activated and cell-to-cell adhesion is strong. (B) Embryo raised in calcium free seawater in which cadherin influence is mitigated.

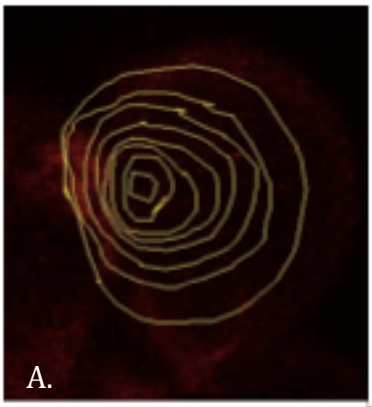
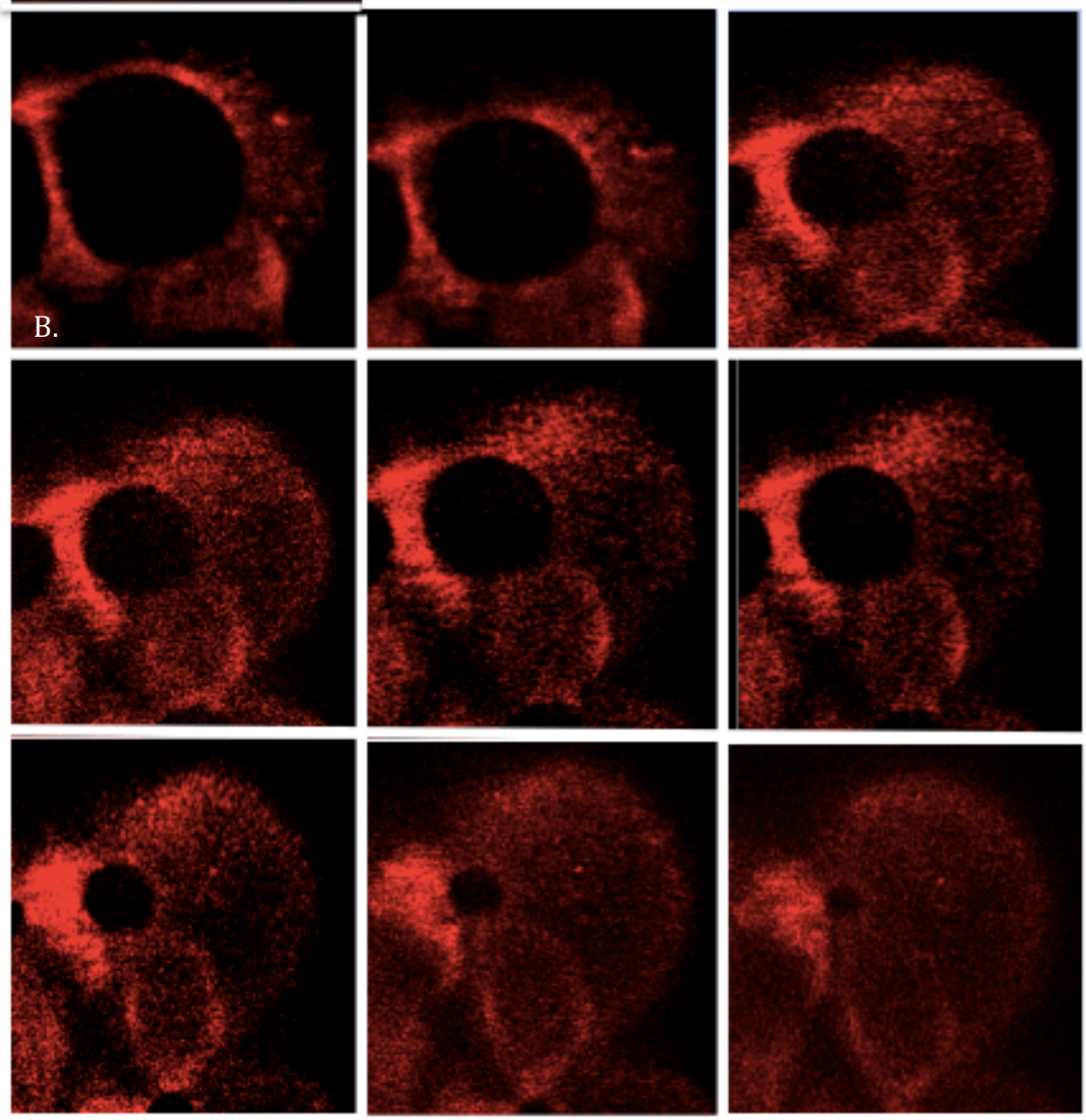


Figure 9. Contractile Ring Shape of *L. Pictus* in Calcium free water. (A) Contractile ring shape was analyzed at 20 seconds intervals during the third division using flourescently labeled plasma membrane from CellMask. Embryos were raised in CaFSW. (B) Ring shape was traced over time for clarity. N= 15



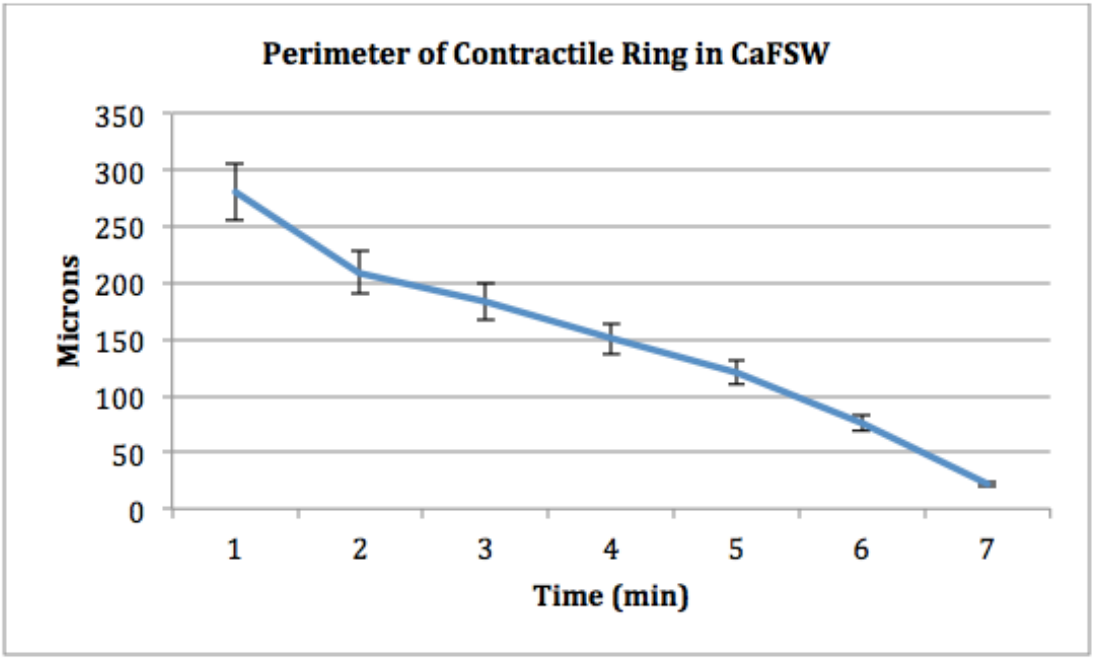


Figure 10. Average perimeter of the contractile ring throughout division. Time interval is one minute.

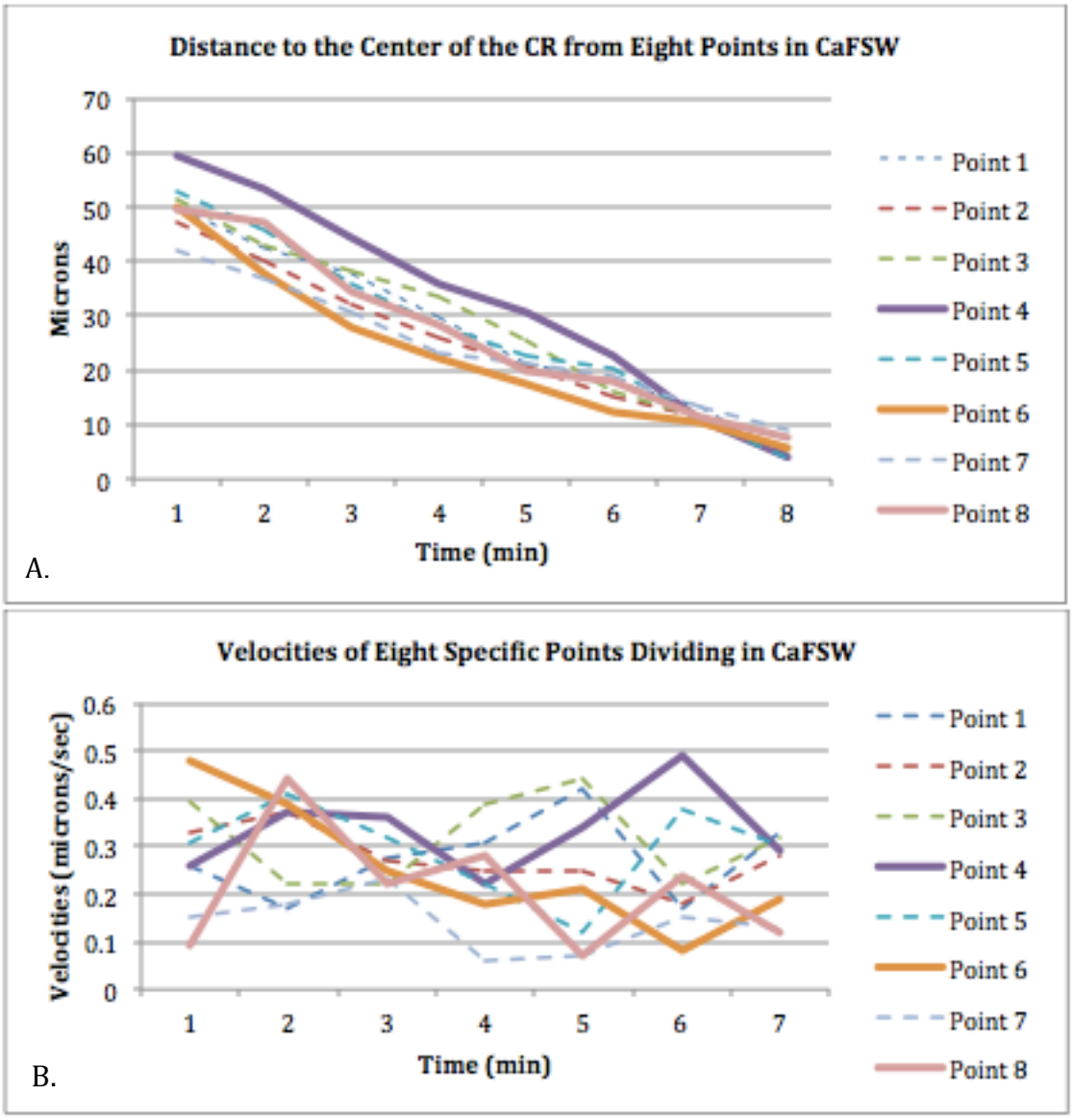


Figure 11. Distance and velocity measurements of contractile ring in CaFSW. Points 4,6, and 8 are highlighted for comparison. (A) Distance from eight specific points to the center of the final visible contractile ring was measure over time. (B) Velocity was measured between each time point. Points were labeled by naming the top middle of the cell "1" and turning clockwise 45º from the center of the contractile ring to label the next initial point. The specific points on the contractile ring were followed by maintaining the angle in relation to the center. N=15.

Contractile Ring Shape During Divisions in Microwells

The contractile ring is forced to begin as a noncircular shape within the four-cell embryo, but the contractile ring can also be manipulated into a noncircular shape through the use of microfabricated chambers. PDMS chambers were used to alter the shape of a fertilized egg prior to the first division, and ultimately the contractile ring.

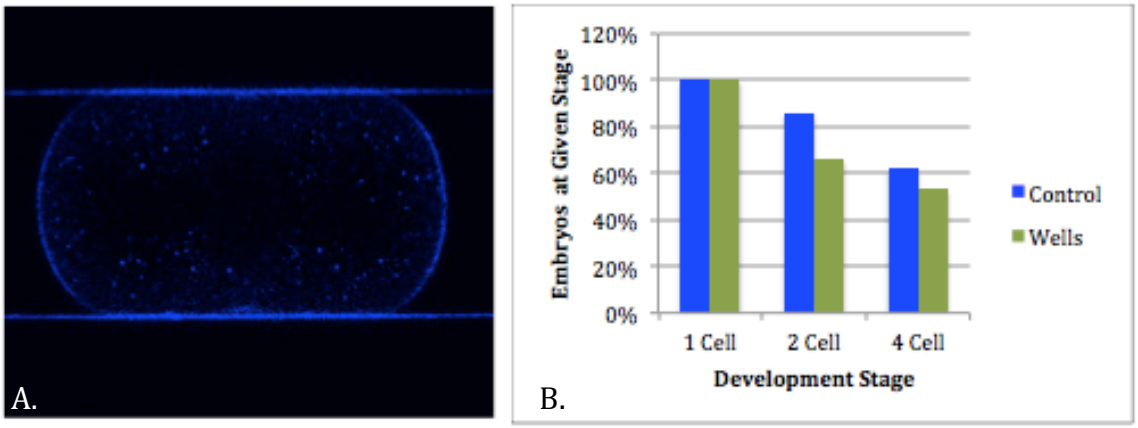


Figure 12. Embryos in microfabricated chambers. (A) Fertilized egg in microfabricated chamber stained with CellMask. (B) Embryo development was tracked over time to determine whether placement in the microfabricated chambers stunted development.

PDMS microfabricated chambers have been widely used in recent years;[41][42] [43] however, cytokinesis has not been directly studied using this technique. To ensure that raising embryos in wells would not adversely affect cytokinesis, percentage of embryos that have undergone division was recorded. There was a slight time delay for divisions, but overall there was no statistical significance between embryos reaching the four-cell stage, but a strong manipulation of the contractile ring was achieved (Fig. 12A, 12B). Embryos were followed to the four-cell stage because cytokinetic failure happens more during the first and second divisions, so an embryo that divided to the four-cell stage has a higher likelihood of being healthy. Shape of the contractile ring was imaged and analyzed at 60-second intervals (Fig. 13). Approximately 10 samples were collected. Velocities were analyzed as

previously described. The contractile ring behaved similarly to the noncircular rings in both the SW and CaFSW (Fig. 13). The method of labeling points stayed the same, but points of interest or furthest outliers of contractile ring circularity are now points 2,4,6, and 8 (Fig. 14). Distance measured from each point to the center of the contractile ring showed various average velocities for each point as the slopes of the lines varied (Fig. 15A). The point velocity analysis showed that the contractile ring became circular before contracting overall (Fig. 15B). Corners of the rectangle move more quickly than the sides to round the ring initially before overall contraction.

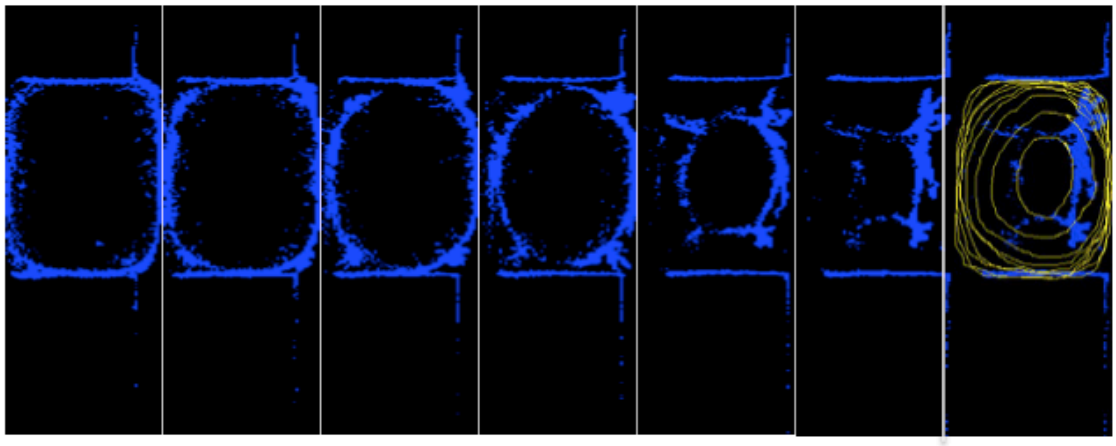


Figure 13. Contractile ring shape of *L. pictus* in microfabricated rectangular chamber. (A) Contractile ring shape was analyzed at 20 seconds intervals during the first division using CellMask to stain the plasma membrane. Z-slices were taken every 1.25 microns and orthogonal veiws were used to visualize ring. (B) Ring shape was traced over time for clarity.

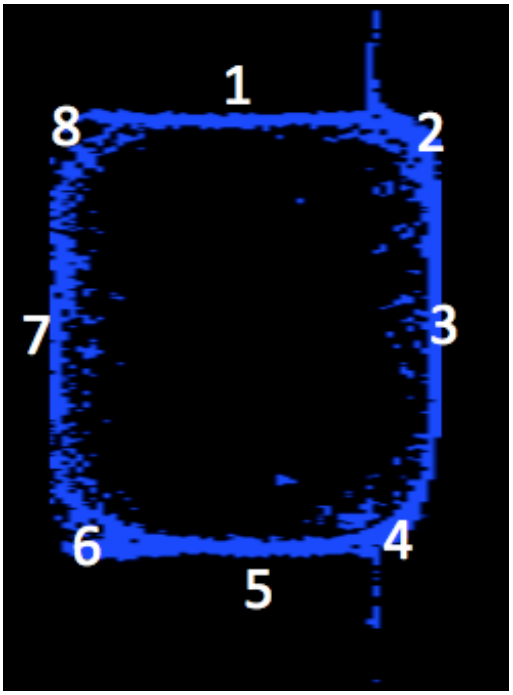


Figure 14. Schematic demonstrating the numbering and tracking of specific points along the contractile ring. Points of interest include 2,4,6, and 8, which lay at the corners of the original shape of the contractile ring.

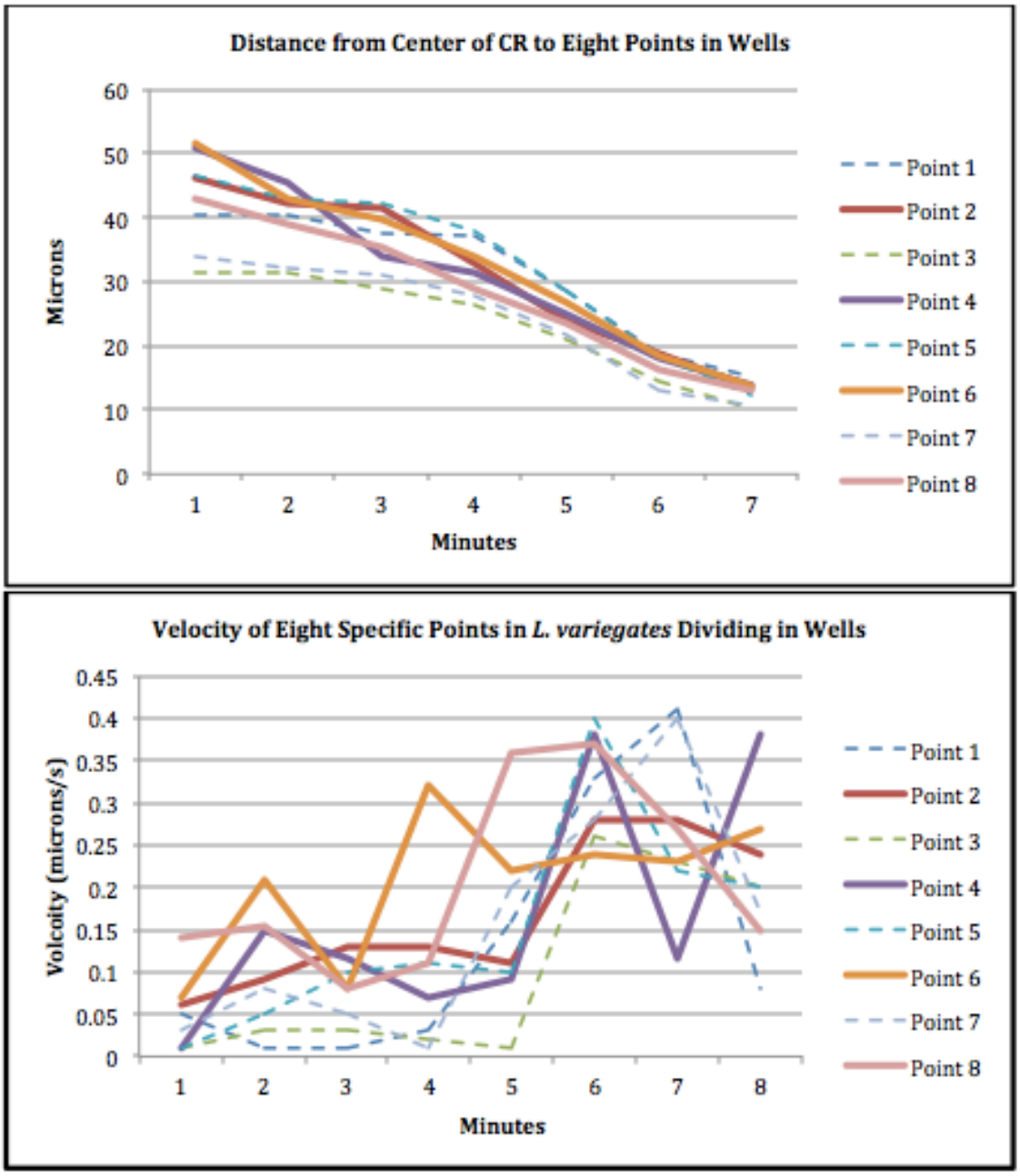


Figure 15. Distance and velocity measurements of contractile ring constrained by microfabricated chambers. (A) Distance from eight specific points to the center of the final visible contractile ring was measure over time. (B) Velocity was measure for movement between each time point to get a closer look. Points were labeled by naming the top middle of the cell "1" and turning clockwise 45º from the center of the contractile ring to label the next initial point. The specific points on the contractile ring were followed by maintaining the angle in relation to the center. Points 2,4,6, and 8 are highlighted as these correspond to the corners of the rectangle. N=10.

Analysis of Actin Localization

Point velocity analysis shows that different parts of the contractile ring are moving at different speeds throughout cytokinesis, with a preference for the furthest or least circular sections moving quickly at first to achieve a more circular contractile ring. For this to happen, there must be a greater force produced by the contractile ring at these points. Recent research has shown that there is little recycling of myosin and force produced by the contractile ring comes from distinct contractile units whereby more force is equivalent to more units being present.

To investigate this idea LifeAct© was microinjected into a single blastomere of a two cell embryo. Embryos were raised in seawater to ensure cadherins were providing the normal cell-to-cell adhesion forces hence requiring a larger degree of force by the contractile ring to counter these adhesion forces. However, when actin distribution was analyzed at the third division from the four-cell stage to the eight-cell stage, no actin localization was seen (Fig. 16B). Actin remained approximately evenly dispersed throughout the contractile ring, suggesting it is not simply a case of more contractile units producing more force. If more contractile units were needed to produce a greater force, it would make sense that actin would be localized initially to the side of the contractile ring pulling against cell-to-cell adhesions.

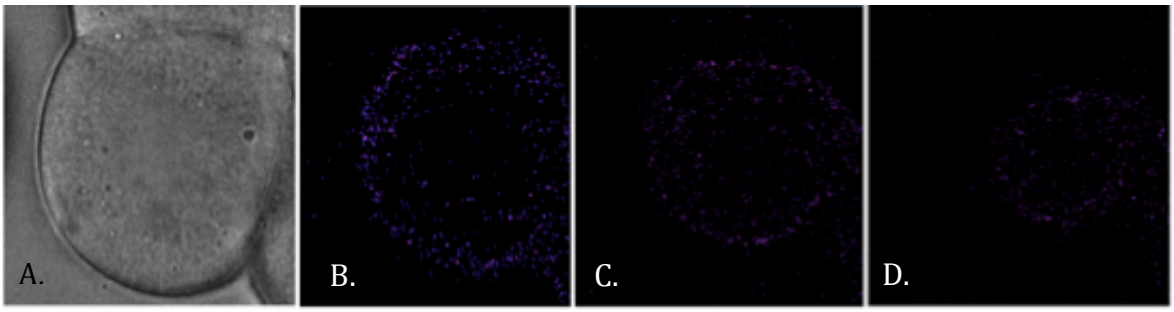


Figure 16. Actin distribution in the contractile ring during the third division in *L. pictus*. (A). Bright field image of a single blastomere in a four-cell embryo raised in seawater injected with LifeAct© Ruby. (B) LifeAct© Ruby injected to visualize actin at (B) t=0 (c) t=150s (D) t=300s.

One alternative possibility is that it is not simply the presence of the contractile units that produces force but the rate of actin exchange within the ring that creates a more dynamic and forceful ring. This could be investigated through a FRAP experiment of the contractile ring in a cell that has been microinjected with LifeAct©.

DISCUSSION

Velocities of specific regions of a noncircular contractile ring show that there first is a rounding of the contractile ring followed by overall constriction of the ring. This holds true in seawater when cadherins provide a significant counter force for the contractile ring. Even in this situation, regions of low circularity have higher initial velocities than the free, circular portions of the ring. This indicates rounding happens before overall constriction. A higher initial velocity of the contractile ring in regions where the ring interacts with cell-to-cell adhesions requires an uneven production of force. If the contractile ring produced an even force around the circumference of the ring, the portions of the contractile ring pulling against cell-to-cell adhesions would have a lower velocity initially then the portions of the contractile ring coming from the free side.

This principle of rounding before overall constriction holds true for contractile rings manipulated by PDMS microfabricated chambers to begin as rectangles. When the contractile ring was artificially manipulated to begin as a rectangle, the corners of the rectangle have a higher initial velocity than the sides of the rectangle, which serves to round the contractile ring. Therefore, the contractile ring ends up circular, but describing the ring simply as circular ignores the unique initial geometry.

The contractile ring appears to create an uneven force in a coordinated manner throughout the ring to deal with initial noncircular or irregular shapes before eventually rounding the ring. The contractile ring may not operate as a homogenous, singular structure but rather a coordination of sections capable of acting uniquely. Both force and geometry of the contractile ring should be investigated by treating the contractile ring not as a single entity but in sections, a novel approach. The contractile ring is not simply circular and studies investigating force production and dynamics, should take such geometry into account.

The mechanism by which cells divide has been studied for decades, but contractile ring shape throughout cytokinesis has been a large gap in research. Geometry can play a role in the need for localized force production, and looking at

the ring through that lens has the potential to illuminate the complex and rearranging molecular components. Investigating the role that geometry can play on contractile ring dynamics can provide a link between biophysical and molecular insights. Force production and molecular components of the ring have both been seen as uniform throughout the ring, but the evolving geometry of the ring suggests there may be unique subsections of the ring that may have differing biophysical and molecular properties.

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