DISTRIBUTION OF MICROFILAMENTS FOLLOWING FERTILIZATION OF DREISSENA BUGENSIS

By

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DISTRIBUTION OF MICROFILAMENTS FOLLOWING FERTILIZATION OF DREISSENA BUGENSIS

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ABSTRACT

Quagga mussels, *Dreissena bugensis* (*D. bugensis*), are an invasive species that are closely related to zebra mussels. In recent years, these mussels have infested many areas due to their ability to reproduce efficiently. This has led to the disruption of native ecosystems, clogged water intake pipes in water treatment plants, damage to ships and boats, and several other consequences. In the United States alone, it is thought to cost more than $200 million a year to deal with these mussels.

Fertilization of *D. bugensis* occurs externally, as the bivalves release their gametes into the water. Following binding of the sperm to the egg, an insemination cone forms that helps facilitate the movement of the sperm nucleus to the egg cortex. After successful incorporation of the sperm nucleus into the egg, mitosis begins; and the spindle fibers form to initiate the first cleavage of the egg following fertilization. This study concentrates on the role of actin and where it is located at specific times following fertilization. This will help us understand the complex process of fertilization while possibly providing a way to slow the spread of *D. bugensis*.

Acknowledgments

I would like to acknowledge Dr. Mike Misamore for working as my research advisor and for guiding me throughout my research. I would like to thank Dr. Matt Chumchal and Dr. David Minter for serving as my senior honors research committee members. I would also like to thank Sarah Barnard for the pictures provided to me from her previous work in the lab.

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Introduction

Dreissena bugensis, better known as the quagga mussel, is an invasive bivalve that has become an increasing problem in the United States. These freshwater bivalves, along with the closely related zebra mussel (*Dreissena polymorpha*), are rapidly spreading throughout the nation's waterways. While *Dreissena bugensis* is indigenous to the Dnieper drainage system in the Ukraine (Mills et al. 1996), the mussel has proven to be effective at invading other parts of the world. Several features of their biology enable them to spread rapidly. These bivalves exhibit broadcast spawning so they release their gametes into the water column for external fertilization. This allows the larvae to be transported, as the animals or the larvae can easily be taken up by the ballast of a ship and transported across the Atlantic to the Great Lakes (Carlton, 1992). Both mussels also exhibit a planktonic veliger stage, where the larvae can stay suspended in water for several weeks and be transported great distances before settling down (Carlton 1993). They also produce byssal threads that allow the adult mussels to attach to hard substrates, such as the hull of a ship (Johnson and Carlton 1996). Their ability to spread to new places and to establish new populations with ease has allowed both of these mussels to become invasive.

Quagga mussels and zebra mussels have both negatively affected the ecosystems into which they were introduced, and they have caused an economic strain on the areas where the mussels proliferate. Both mussels were introduced into the Great Lakes in the mid to late 1980's, where water quality rapidly diminished following the initial colonization by the mussels (Fahnenstiel et al, 1995). Quagga mussels are very effective at filter feeding so they digest a significant amount of phytoplankton, on which

zooplankton feed (Synder et al. 1997). Their introduction effectively changes an entire ecosystem. They are also able to accumulate pollutants at very high concentrations within the tissues that can be released into the water that we drink (Snyder et al. 1997). The economic strain comes from their ability to attach to hard surfaces. They can easily colonize water intake structures of power and water treatment plants and effectively stop water flow (Snyder et al. 1997). The estimated cost of control is around $200 million a year (U.S. EPA). Having a better understanding of their biology can aid in developing mechanisms for controlling their spread.

Zebra and quagga mussels are also good model organisms for studying development. They produce a large number of gametes which can be induced to spawn by exposure to serotonin (Ram et al. 1993). The gametes are released into the water column where fertilization and subsequent larval development occurs making it easy to observe these processes. Additionally, the cytoplasm of the eggs is very clear which allows intracellular events such as sperm inside the egg to be readily observed (Misamore et al. 1996). While several studies have focused on various aspects of zebra mussel reproduction (Fong et al 1994, Miller et al 1994, Luetjens, 1995, Misamore et al 1996, 2000, Fallis et al 2006), few studies have focused on quagga mussel reproduction. Therefore, in this project, we are addressing the role of the cytoskeleton in early quagga mussel development. Specifically, we will address the distribution of actin at various points in the early development of quagga mussels. We will show the localization of actin with sperm and unfertilized eggs during key developmental features until the first cleavage of the egg. Descriptions will include actin localization with the sperm, unfertilized egg, fertilization cone, polar body, and cleavage furrow.

Materials and Methods

Specimens of *Dreissena polymorpha* were collected from Lake Mojave by the National Park Service and transported to TCU, where they were maintained at 9 °C in a 55-gal aquarium containing artificial pond water (PW). Animals were fed Shellfish diet 1800 (Reed Mariculture) twice weekly and a 30% water change once per week. Prior to spawning, the mussels were rinsed and isolated in individual specimen cups filled with cold PW and allowed to warm to room temperature for 24-48 hours. This gradual warming helped enhance subsequent spawning and also ensured any gametes present prior to isolation were dead at the beginning of the experiment.

Spawning was induced by external exposure of animals to 2×10^{-4} M serotonin (5-hydroxytryptamine) for 1 hour (Ram et al. 1993, Misamore et al., 2000). Males typically released their gametes around 30 minutes post treatment of serotonin, while females released their gametes around an hour after treatment. Females were transferred to 50-ml crystallizing dishes to complete spawning. Following spawning, eggs were inseminated with 100 ul of sperm.

Samples of the specimens were taken at specific time points following insemination. The time points used were 5, 10, 15, 30, 45, and 60 minutes post insemination so that we could see the different benchmarks of development following fertilization. The fixative used was 3.2% paraformaldehyde in Mussel Buffer (5 mM TAPS, 0.8 mM NaCl, 0.145 mM KCl, 1.8 mM Na_2SO_4, 0.887 mM $MgSO_4 \cdot 7H_2O$, 1.32 mM $NaHCO_3$, 1.19 mM $CaCl_2 \cdot 7H_2O$, pH 7.6). This is a modification of previous mussel fixatives (Misamore et al. 2000) minus methanol. The absence of methanol was to avoid the disruption of phalloidin staining due to methanol fixation. Acetone was

incorporated into the fix so that the membrane would be more permeable to both the fix itself and the actin stain that we would eventually be using. Each sample was washed two times with Mussel Buffer (Misamore et al. 2000) before further processing with the actin stain.

Staining for actin using Alexa Fluor® 488 Phalloidin (Molecular Probes/Life technologies, Inc) was carried out as follows. Eggs were washed twice with 100% acetone, which helped permeabilize the egg so that the stain could effectively enter the egg. Samples were stained with 7 μl of stock Phalloidin solution to 250 mL of a solution eggs. Each sample was allowed to stain for 10-15 minutes; then it was washed twice with Mussel Buffer. Phalloidin stained samples were placed on a microscope slide with silicone grease posts to support the coverslip and prevent sample damage. A drop of Slow fade gold anti-fade agent containing DAPI was added to the samples to stain DNA and help prolong the actin stain. Fluorescent microscopy was performed on a Zeiss Axiovert microscope with phase contrast optics. A Zeiss Axiocam camera and axiovision software were used for digital image capture. Final image processing was done with Adobe Photoshop.

Results

Sperm

The sperm of *D. bugensis* contain three general regions: a head, a midpiece, and a flagellum (Walker et al. 1996). The head region contains the nucleus of the sperm and a conical acrosome with an axial rod within the acrosome (Misamore, 2000). The midpiece contains four mitochondria. The flagellum is the tail of the sperm which beats in order to provide movement. Within these three regions, actin was only observed in the

tip of the sperm at the acrosomal region (Figure 1). Actin appears as a prominent acrosomal rod extending anteriorly from the nucleus toward the end of the acrosome (Figure 2).

Egg

Eggs from *D. bugensis* were uniformly round. The eggs had not been fertilized yet so they were still arrested in metaphase I. Chromosomes were still decondensed within the nucleus, and no polar bodies had formed yet. The outside of the eggs were covered with a layer of microvilli which help fertilization occur (Figure 3a). Fluorescent staining showed that actin was only seen along the surface of the egg associated with the microvilli (Figure 3b).

Fertilization

Following insemination, sperm binds to the surface of the egg. The actin-filled acrosomal filament is in direct contact with the egg surface. After binding, the sperm is gradually drawn into the egg cytoplasm (Figure 4). An evident fertilization cone forms around the incorporating sperm. The fertilization cone helps incorporate the sperm into the cytoplasm of the egg. The fertilization cone appears around three minutes post insemination (PI) and persists until about ten minutes PI. Actin labeling shows that the fertilization cone is composed of actin that surrounds the fertilizing sperm (Figure 5).

Around 5-7 minutes PI, the first polar body begins to form as the egg is no longer arrested in metaphase I. Observations of the polar bodies under fluorescence shows that the polar body is full of DNA, and a bundle of actin lies at the site of the cleavage furrow between the egg and polar body (Figure 6).

Pronuclei form around 30 minutes PI, and the DNA within them starts to condense and migrate toward one another in preparation for fusion. Shortly after pronuclear co-mingling, a sperm aster evolves into the mitotic spindle in preparation for its first round of mitotic division. These events are observable due to the transparent nature of the egg cytoplasm. However, no discernible actin was detected during pronuclear formation or migration.

Following separation of the chromosomes during karyokinesis of first cleavage, cytokinesis is able to occur. At around 60 - 70 minutes PI, the cleavage furrow begins to form to separate the egg into two separate cells. Fluorescence shows that significant amounts of actin are localized within the cleavage furrow (Figure 7). No actin formation was associated with the nuclei of the new daughter cells.

Discussion

Fertilization of *Dreissena bugensis* follows the fertilization patterns of other organisms, and the distribution of actin is the same as other species. In the following, we will detail where actin is located and the associated function with each of the fertilization events.

Sperm

Of the three regions of the sperm, actin was only observed within the head region, specifically within the acrosome (Figure 1 and 2) (Lora-Lamiea et al, 1986). When the sperm binds the egg, the acrosome reaction occurs. The acrosome opens up and exposes the axial rod which binds to the microvilli of the eggs in order to facilitate passage through the vitelline coat, and subsequent fusion to the egg. The acrosome reaction also

exposes the inner acrosomal membrane which covers the axial rod. In sea urchins, this inner acrosomal membrane contains proteins essential for sperm binding (Vacquier and Moy, 1977). Additionally, the acrosome reaction releases materials necessary for sperm-egg binding, though that has not been fully investigated (Hylander et al, 1977). The actin filaments are necessary for the acrosome reaction and subsequent insemination. In many species including other bivalves, the acrosomal filament elongates or extends outward during the acrosome reaction (Hylander et al, 1977). In zebra mussels, the acrosomal filament is of a fixed length and does not further elongate during fertilization (Misamore et al. 1996). Here, we show that the acrosomal filament in quagga mussels consists of actin and does not appear to elongate during the acrosomal reaction. These actin labeling results confirm the presence of actin as a major component of the quagga mussel acrosomal filament suggested by electron micrograph images (Walker et al. 1996).

Egg

D. bugensis eggs exhibit the same general morphology as other bivalves, such as Zebra mussels. The eggs themselves are arrested in Metaphase I, where they remain until fertilization. The DNA remains condensed until fertilization occurs. At this time, the egg continues its meiotic divisions. Actin lines the outer surface of the egg which corresponds with numerous microvilli along the egg surface (Figure 3). This further emphasizes the presence of actin-containing microvilli on the surface of the eggs. Actin-containing microvilli are found in many other species, including sea urchin eggs (Burgess et al, 1977) and other bivalve species (Hylander and Summers, 1977). Electron microscopic observations of zebra mussel eggs suggest the presence of actin-filled microvilli, but actin-specific labeling to confirm this has yet to be done. No observable

actin was detected within the egg cytoplasm. However, background fluorescence may be obscuring low levels of actin. Additional work to reduce inherent background fluorescence should verify the presence or absence of cytoplasmic actin.

Post Insemination

The sperm binds almost immediately after insemination occurs. The acrosomal filament of the sperm binds with the microvilli of the egg, with the sperm binding perpendicular to the egg. Once the sperm has come into contact with the egg, the acrosomal reaction occurs. In sea urchins, the acrosome reaction process involves actin elongation to form the axial rod within seconds following binding (Tilney et al, 1972). However, Zebra mussel sperm, which exhibit a physiology similar to *D. Bugensis*, have a preformed axial rod that is long enough to connect to the egg surface without additional polymerization of the axial rod (Misamore et al, 2000). The axial rod of *Bugensis* is made of actin, which allows it to interact with the actin within the microvilli of the egg.

The first notable event leading to incorporation of the sperm is the formation of the fertilization, or insemination, cone. This structure on the egg surface forms between the sperm and the egg as the sperm is drawn into the egg cytoplasm. It has been reported that the fertilization cone forms around 2 minutes PI and persists until around 10 minutes PI, with the cone exhibiting the most robust amount of actin around 7 minutes PI during sea urchin fertilization (Tilney et al, 1980). *D. Bugensis* eggs seem to follow a similar timeline. As expected, examination of the fertilization cone shows the presence of actin filaments (Kyozuka et al, 1988). DAPI staining also shows DNA within the fertilization cone. When treated with an actin inhibitor, the fertilization cone is unable to develop (Misamore et al, 2000). It is thought that the fertilization cone is necessary for sperm

incorporation, as actin inhibitors are able to prevent fertilization even after sperm binding but prior to sperm entry. Therefore, the fertilization cone is a structure that likely helps bring the sperm into the cortex of the egg, which is supported by the strong correlation between actin and DNA within the insemination cone (Figure 5). However, more research is needed to determine the actual function of the insemination cone.

Once the sperm is inside the egg, it appears that actin surrounds the DNA (Figure 4). This is most likely to aid in the mobility of the sperm DNA so that the DNA can move across the cytoplasm and become incorporated into the egg. There is still a darker band of actin around the tip probably corresponding to the remnants of the acrosome. However, more studies are needed to see the actual function of actin for sperm incorporation.

Around 10 minutes PI, the first polar body can be seen. The polar body is the result of the egg proceeding through its meiotic divisions. When the egg goes through meiosis, it has an uneven division in which the egg retains the majority of the cytoplasm from the polar body, while the polar body receives all of the DNA. For cleavage to occur, a contractile ring must form between the two cells. This ring, which is made of actin, contracts until it pinches off the cells, and two daughter cells are left. Fluorescence microscopy shows that the polar body is mostly DNA, with the border between the egg and the polar body showing the presence of actin (Figure 6). This supports the idea that the polar body just contains DNA, and actin is needed to drive cytokinesis. Therefore, we should expect actin to be seen between the two cells during any type of division.

Sperm aster and the formation of the mitotic spindle is seen around 50-60 minutes PI. The sperm contributes the spindle for the first cleavage in the form of the sperm

aster. The sperm aster in invertebrates consists of the centrosome and the cortex, which consists of microtubules that emanate from the centrosome (Longo, 1976). The mitotic spindle binds to the condensed DNA and separates the DNA by pulling it apart. However, we were not able to observe any actin in this process. This may be because there is no actin to detect, as the main cytoskeletal elements involved are microtubules. A lack of actin labeling may be due to background fluorescence blocking our view of the actin. However, sperm aster formation has been well studied in other species which do not include actin in the process. Thus, it is reasonable to assume that the same process will drive the first cell division.

The first mitotic division is seen around 60-70 minutes PI. The spindle has formed at this point and has separated the chromosomes. By this time, the DNA has decondensed into the respective nuclei. As expected, actin can be seen lining the cleavage furrow in a fashion similar to the polar bodies (Figure 7). Actin is responsible for cleavage in the same process that was observed with the polar bodies.

Overall, the distribution of actin throughout fertilization follows what we would expect from other species and what has been suggested for zebra mussels. Actin was shown to be prominent in the acrosomal rod of the sperm and the microvilli of the eggs. It was prevalent in the fertilization cone during sperm incorporation and in the cleavage furrow during polar body formation and first cleavage. Since actin is prevalent thoughout early development and is shown to be necessary for fertilization, it could be useful as a potential target as a method to control the rapid spread of Quagga mussels. This study provides the first detailed distribution of actin during fertilization in a dreissenid mussel.

FIGURES

Figure 1 Sperm

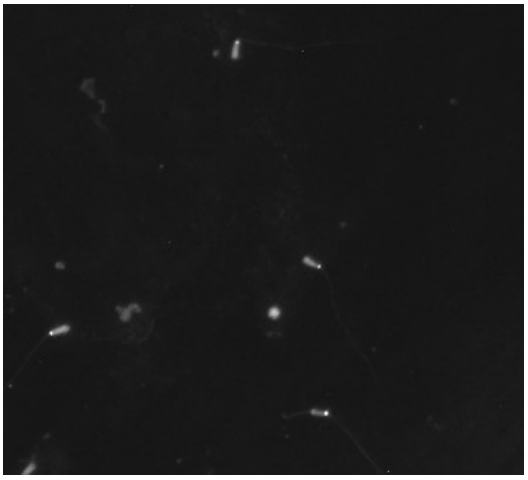

Figure 1. Light micrograph with phase contrast showing the distribution of actin within unbound sperm. Note the dark band of actin corresponding to the acrosome.

Figure 2 Sperm TEM

Figure 2. Transmission electron micrograph of sperm. Note the acrosome with the acrosomal rod at the tip of the sperm.

Figure 3a Egg Cortex

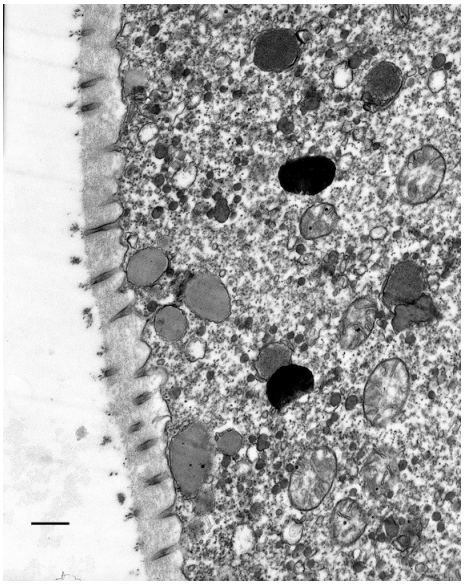

Figure 3a. Transmission Electron image of *D. Bugensis* unfertilized egg. Note the microvilli lining the outer surface of the egg (Photo by: Sarah Barnard).

Figure 3b Unfertilized egg

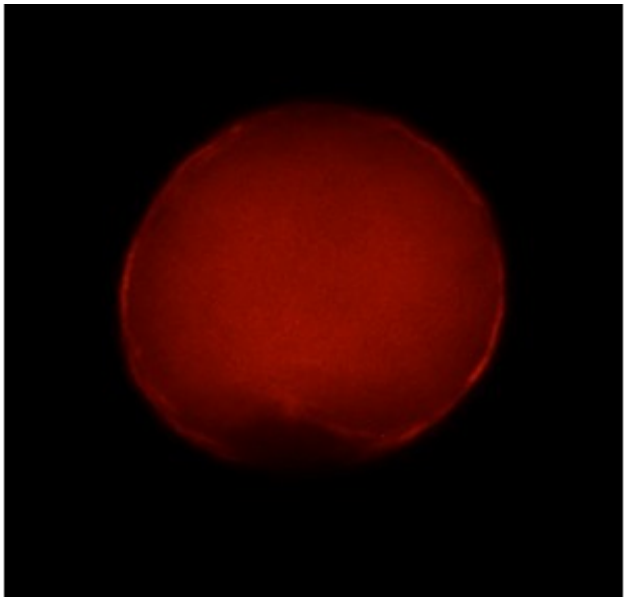

Figure 3b. Fluorescence micrograph showing the distribution of actin in a unfertilized egg. Note the actin surrounding the egg corresponding to the microvilli.

Figure 4 Sperm inside of Egg

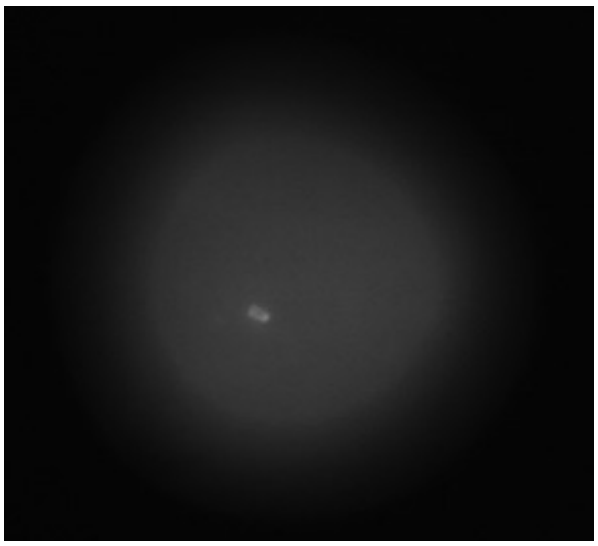

Figure 4 Fluorescent Micrograph of actin. Note the sperm inside the egg with the acrosome still intact.

Figure 5 Fertilization Cone

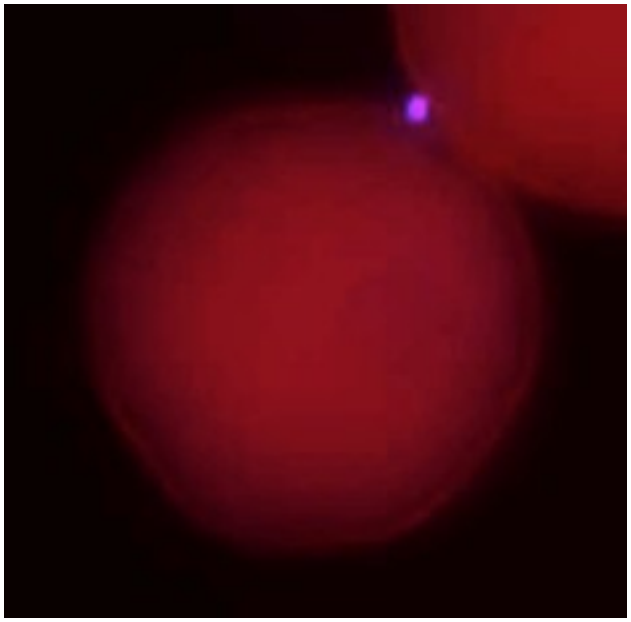

Figure 5 Fluorescent Micrograph of the fertilization cone, with the DNA and actin images superimposed over the other, with blue corresponding to DNA and red corresponding to actin. Note the purple region that corresponds to the fertilization cone.

Figure 6 Polar Body

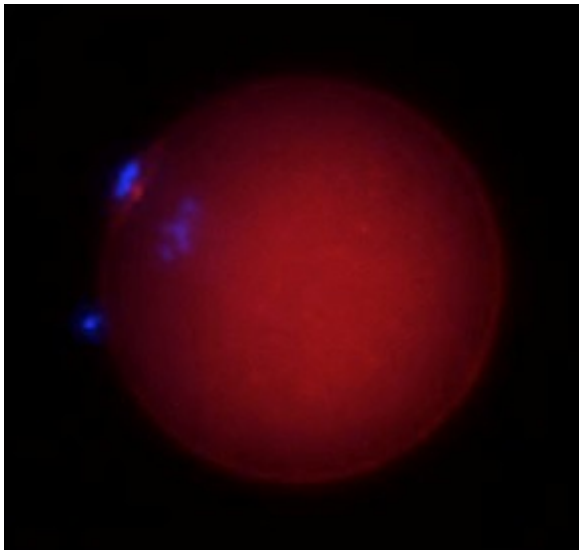

Figure 6 Fluorescent Micrograph of the first polar body. DNA is shown in blue, and actin is shown in red. Note the DNA within the polar body. Note the actin at cleavage furrow between the egg and the polar body.

Figure 7 Egg Division

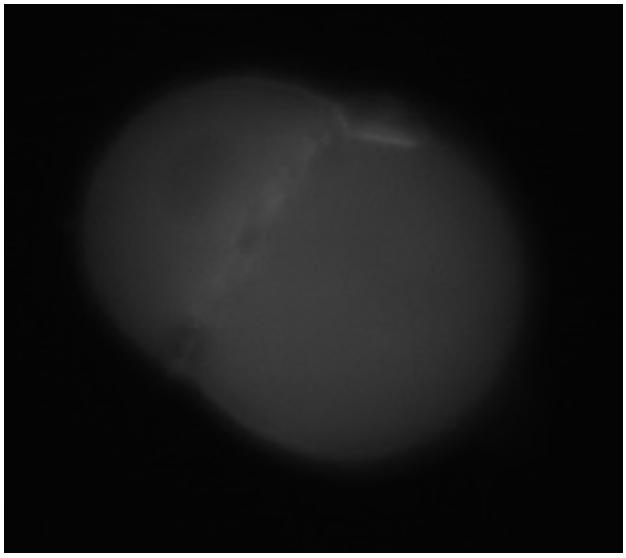

Figure 7 Fluorescent Micrograph of actin distribution in the first division of the cell. Note the bright areas corresponding to actin between the two daughter cells. Note the actin between the egg and the polar body.

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