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Amanda Bastien April 15, 2015

Development of a Voltage Sensor Tool, *mermaid-2*, for Use During Fertilization in *Caenorhabditis elegans*

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An abstract of

A thesis submitted to the Faculty of Emory College of Arts and Sciences

Of Emory University in partial fulfillment

Of the requirements of the degree of

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Department of Biology

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Abstract

Development of a Voltage Sensor Tool, *mermaid-2*, for Use During Fertilization in *Caenorhabditis elegans*

By Amanda Bastien

My lab studies *Caenorhabditis elegans,* which is a great model organism for studying fertilization. Many different fertilization defective mutants have been identified and now there is a need for new tools to analyze the phenotoypes of these mutants. In particular, we are interested in studying the *spe-9* class of mutants. The *spe-9* class includes mutants in eight genes, all of which are able to complete spermatogenesis and appear indistinguishable from wild type, except that they cannot fertilize an oocyte when they contact it. All eight genes cause the same fertilization-defective phenotype and, currently, there is no way to phenotypically distinguish them from each other. My goal was to develop a voltage sensor tool to use *in vivo* in *Caenorhabditis elegans* to help determine if there is a voltage change during fertilization. Previously, fluorescent reporters have been used in a wide variety of organisms to measure voltage changes *in vivo* (TSUTSUI *et al.* 2008; 2013). My thesis is centered on creating a *mermaid-2* gene that could be used in the *Caenorhabditis elegans* germline*,* microinjecting the plasmid, confirming its presence, and detecting *mermaid*-2 expression. However, the expression of *mermaid-2* is still being investigated in current transgenic lines.

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Acknowledgements

I would like to thank my advisor, Dr. Steven L'Hernault for his constant support and mentorship over the past two years as well as my committee members, Dr. Guy Benian and Dr. Levi Morran, for their insight during this project. I would also like to thank Elizabeth Gleason for her continuous guidance and patience everyday and Dr. Candice Elam and Huiping Ling for their help and advice as well. Lastly, I would like to thank my family, especially my parents, and all of my friends who listened to my rants about injections and worm gonads over the past year!

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CHAPTER I

General Introduction

Introduction to Fertilization and Polyspermy

Fertilization is defined by the process where a female gamete, an egg, and a male gamete, sperm, come together to create a new individual with genetic information from both parents (GILBERT 2000). Fertilization in mammalian and nonmammalian organisms share many common features (WASSARMAN 1999). One similarity that is observed is that simultaneous or nearly simultaneous fertilization of an egg by two or more sperm (polyspermy) is likely to be lethal in most organisms (Frank 2000). Furthermore, many organisms have developed a block to polyspermy.

In all animals studied thus far, fertilization causes a change in calcium dynamics within the oocyte (SAMUEL *et al.* 2001). However, the calcium fluctuations are different across animal species. Some animals have a single calcium change while others have multiple fluctuations (SAMUEL *et al.* 2001).

Fertilization in Model Organisms including the Sea Urchin

The sea urchin has been a highly studied model organism for understanding fertilization. A plasma membrane depolarization normally occurs during fertilization when a sperm enters the sea urchin egg. Furthermore, evidence suggests this voltage change in the plasma membrane occurs to prevent polyspermy for a short amount of time and happens very quickly (LYNN AND CHAMBERS 1984). The ion concentration difference is most significant for sodium and potassium ions (more potassium inside of the cell and more sodium outside of the cell) causing the resting membrane potential to be about -70 mV. Once one sperm binds to the egg, the egg membrane potential depolarizes and sodium moves into the egg causing the membrane potential to rise to

+20 mV. At this point, no other sperm can bind (LONGO *et al.* 1986). It is still uncertain if the voltage change is a result of sperm binding to the egg or the actual act of fertilization. Fertilization can be prevented in entirety when the egg is kept at a positive voltage (JAFFE 1976). This block in fertilization is also seen in frogs, and also in a study with nermetean worm, *Cerebratulus*. In *Cerebratulus* there is a change in membrane potential levels from -66 mV to +43 mV at fertilization. These results suggest that an electrically-mediated polyspermy block at the egg plasma membrane level occurs for approximately an hour after the occurrence of fertilization (CROSS AND ELINSON 1980; KLINE *et al.* 1985).

Many animals, including the sea urchin, have a second mechanism to prevent polyspermy. Unlike the mechanism described above, this is a slow non-potential shift, accomplished by the cortical granule reaction (CARROLL AND EPEL 1975). Evidence suggests that a calcium ion wave initiates the cortical granule exocytosis (EISEN *et al.* 1984; HAMAGUCHI AND HIRAMOTO 1981). The slow block involves the transformation of the viteline envelope to a physical barrier that is impenetrable to sperm. Underneath the vieteline envelope is where the cortical granules are located. The sea urchin fertilization envelope is assembled from the secreted cortical granule components on the vitelline layer (WONG AND WESSEL 2004). The vitelline layer contains ~25 different proteins by gel electrophoresis (GACHE *et al.* 1983). This envelope prevents other sperm surrounding the egg from entering over a long period of time.

In humans, studies strongly suggest a block in polyspermy during fertilization as well. In many mammals, including humans, a large quantity of sperm enters the female reproductive tract. However, most of these sperm are eliminated from the female

reproductive tract before reaching the fallopian tube where fertilization occurs (YANAGIMACHI 1994). The block in polyspermy is thought to take place at the zona pellucida after fertilization. However, this complex mechanism of polyspermy block is still being investigated and is not fully understood (MIO *et al.* 2012).

Overall the molecular requirements for single sperm fertilization are similar, which suggests there could be a common ancestral block to polyspermy (WONG AND WESSEL 2005). In general there is a "fast block" to polyspermy occurring quickly, changing the voltage potential of the plasma membrane and the second " slow block" involving modification of the egg's extracellular matrix. However, as seen in the sea urchins, these events do not have to be mutually exclusive.

Chapter II

Introduction to *Caenorhabditis elegans*

Overview *of Caenorhabditis elegans*

Caenorhabditis elegans (*C. elegans)* is a simple, multicellular species with defined developmental patterns. In 1965, Sydney Brenner chose *C. elegans* as a model organism to study animal development (BRENNER 1974). Thirty-three years later, scientists sequenced its entire genome (ABBOTT et al. 1998). *C. elegans* has a short lifespan, short reproductive cycle, and is easy to grow in the laboratory, making experimentation quick and inexpensive (BOLKER 1995). Even though *C. elegans* contains less than 1000 somatic cells, these worms have diverse cell types and have a fully functioning nervous system (WHITE *et al.* 1986). Its genome is approximately 30 times smaller than the size of the human genome, but there is 40% sequence homology between the two species (LAI *et al.* 2000).

Many organisms have a male or female sex determination, but *C. elegans* is unusual in that its two sexes are hermaphrodite (predominant) and male. Hermaphrodites produce approximately 300 sperm during the fourth larval stage, which are stored in the spermatheca. Once the hermaphrodites become young adults, spermatogenesis stops and oogenesis begins (HIRSH *et al.* 1976). Although hermaphrodites can self-fertilize, they are able to mate with males. Males develop by spontaneous non-disjunction of the X chromosome in the hermaphrodite germline at 0.1% frequency or can arise after a male mates with a hermaphrodite at 50% frequency (HODGKIN *et al.* 1979). Males are smaller in size as compared to hermaphrodites and the only gamete they are capable of making is sperm. However, male sperm are 50% larger and can outcompete hermaphrodite sperm in the struggle to fertilize an oocyte (LAMUNYON AND WARD 1998). There are several sex-specific differences between

males and hermaphrodites, but the general body plan and most of the structures except for the gonad are identical (SULSTON AND HORVITZ 1977).

Male germline

In males, there are three important steps to gametogenesis: spermatogenesis, spermiogenesis, and fertilization. While undergoing meiosis I during spermatogenesis, a primary spermatocyte generates two secondary spermatocytes. The two secondary spermatocytes then undergo meiosis II where haploid spermatids bud from a residual body (WARD *et al.* 1981). Spermatogenesis can occur *in vitro,* where spermatocytes are released from males and can differentiate into spermatids in chemically defined medium (WARD *et al.* 1981). Spermiogenesis is the activation of the mature spermatid into a spermatozoon, which has a psuedopod. This activation can also be seen *in vitro* using any one of several chemical activators, which include the ionophore monesin (NELSON AND WARD 1980), proteases (Pronase or trypsin), triethanolamine (TEA) (WARD *et al.* 1983) or Zn^{++} (LIU *et al.* 2013). Sperm activated with TEA, but not Pronase, are known to be competent to fertilize oocytes by artificial insemination (LAMUNYON AND WARD 1994).

Fertilization in *C. elegans* hermaphrodites

Despite having amoeboid spermatozoa, which lack an acrosome and flagellum, *C. elegans* spermatozoa are able to navigate the hermaphrodite reproductive system to find the egg in the spermatheca and complete fertilization. The absence of an acrosome may be due to the oocyte not having a substantial coat (WARD AND CARREL 1979). However, the reproductive tract of the hermaphrodite (Figure 1) is analogous to that of the reproductive tract of a mammal. The proximal gonad, spermatheca, and uterus of

adult hermaphrodites share similar roles to those of a mammal's ovary, oviduct, and uterus, respectively (NISHIMURA AND L'HERNAULT 2010).

In the adult hermaphrodite, contractions of the oviduct pushes the oocyte into the spermatheca, where sperm are stored, leading to fertilization (WARD AND CARREL 1979). When mating a hermaphrodite with a male, male-derived sperm enters the hermaphrodite reproductive tract during copulation through the vulva and then travel to the spermatheca to fertilize an egg (L'HERNAULT 2006). There is a unknown block to polyspermy as one oocyte is fertilized by solely one sperm (WARD AND CARREL 1979).

During fertilization in *C. elegans,* calcium signals have been monitored by the use of calcium fluorescence indicator dyes that were directly injected into the cytosol of the oocytes. When an oocyte enters the spermatheca, this is correlated with a rise in free Ca^{++} ions within the oocyte (SAMUEL *et al.* 2001). The point of sperm entry sets up the anterior-posterior axis of the embryo and Ca^{++} fluxes may also be involved in this process (GOLDSTEIN AND HIRD 1996). All of these phenomena are currently being studied in more detail.

spe-9: Fertilization-defective mutants

Mutations in the *spe-9* gene cause worms to produce sperm with normal morphology and motility but they cannot fertilize an oocyte even when in contact with it (L'Hernault 1988). The *spe-9* gene encodes a sperm-specific transmembrane protein with 10 epidermal growth factor-like repeats in its extracellular domain. In the EGF repeats, mutations have various effects on fertilization; some defects include completely inactive *spe-9* function or defects seen at different temperatures (SINGSON *et al.* 1998). These *spe-9* epidermal growth factor-like repeats are similar to those seen in the Notch

family of transmembrane receptors and ligands (PUTIRI *et al.* 2004). In many organisms, the Notch pathway participates in cell to cell signaling during cell differentiation (ARTAVANIS-TSAKONAS *et al.* 1999). Like Notch, perhaps the *spe-9* encoded protein functions in specialized cell-to-cell interactions, but for *spe-9,* in relationship to fertilization (see Figure 2). This role may include involvement in gamete identification, attachment, signaling, and fusion (SINGSON *et al.* 1998). A list of known *spe-9* class mutants can be found in Supplemental Table 1, but it is predicted that there are more mutants to be identified (NISHIMURA AND L'HERNAULT 2010). In other nematode species, the activated sperm's pseudopod makes contact with the oocyte's surface and the gamete membranes subsequently fuse (FOOR 1968) .

CHAPTER III

Introduction to Project

Goal of Project:

The goal of this project was to develop tools to analyze phenotypes of fertilization-defective mutants in the *spe-9* class (NISHIMURA AND L'HERNAULT 2010). The *mermaid-2* protein is a fluorescent reporter developed to measure voltage changes *in vivo* and it is applicable to a wide variety of organisms. Furthermore, the *mermaid-2* protein is a fluorescence resonance energy transfer (FRET) reporter and it changes from producing a green signal to producing a yellow signal when membrane voltage potential rises (TSUTSUI *et al.* 2013). My goals are to use a transgenically expressed *mermaid-2* optimized for *C. elegans* to test if there is a detectable voltage change in oocytes during *C. elegans* fertilization.

Introduction to MosSCI & Transgenesis

The ability to manipulate the genetic composition of animals has become a key component in today's research. The most widespread example is the tagging of proteins with jellyfish-derived green fluorescent protein (SNAPP 2009). Other examples include inserting a foreign gene into a chromosome (AJF *et al.* 2000). A transgenic technique called Mos1-mediated Single Copy Insertion (MosSCI) is commonly used in *C. elegans.* The excision of a Mos1 transposon creates a double-strand break in the noncoding portion of DNA at a specific chromosomal locus. The cell can then repair this site by copying DNA from an extra-chromosomal template into the chromosome (FROKJAER-JENSEN *et al.* 2008). If no recombination occurs, the injected plasmid can still be maintained in the cell as a stable extra-chromosomal array. During meiosis and mitosis in *C. elegans*, extra-chromosomal arrays are replicated and frequently passed onto daughter cells (MELLO *et al.* 1991).

Transgenesis in *C. elegans* involves microinjection of a desired construct, usually in a plasmid vector, together with a reporter plasmid and subsequently selecting for the rare animal that stably expresses the reporter construct. In most cases, the presence of the reporter plasmid indicates that the desired construct is also present, but this must be experimentally shown to be true (MELLO *et al.* 1991).

The *mermaid-2* Voltage Sensor

mermaid-2 (*mer-2*) is a tripartite cDNA plasmid that encodes one cyan and one yellow coral fluorescent protein-separated by a voltage sensor. The voltage sensor is a phosphatase from the tunicate *Ciona intestinalis* (TSUTSUI et al. 2013). The three dimensional structure of the phosphatase protein is exquisitely sensitive to cellular voltage potential and, at negative voltage potential, *mer-2* expression results in a 483 nm cyan signal when a cell is illuminated with 438 nm (violet) light (see Figure 4). When the membrane potential increases, the tripartite protein folds so as to bring the cyan and yellow coral fluorescent proteins in close proximity. This close proximity allows fluorescence resonance energy transfer (FRET) between the cyan donor and the 547 nm yellow acceptor, resulting in a yellow signal (see Figure 5).

The energy transfer from the excited donor to acceptor is dependent on the distance (within the so-called Förster radius) between the pair of proteins involved in FRET, which are thought to be ~10-100 Å apart (FÖRSTER 1965). Resonance transfer behavior is similar to coupled oscillators, whereas radiative energy requires absorption and emission of a photon (FÖRSTER 1965). FRET has been used for quantifying structure, conformation changes, and as a tool for evaluating biochemical events (LAKOWICZ *et al.* 1990; HEYDUK 2002; BUNT AND WOUTERS 2004; PARSONS *et al.* 2004).

The use of FRET between CFP and YFP has been a common method to monitor protein-protein interactions (TSIEN AND MIYAWAK 1998).

Previous use of *mermaid-2*

The *mermaid* (*mer*) and its improved successor *mermaid-2* (*mer*-2) can serve as biosensors for voltage change in cells from a variety of different species. For example, *mer* expressed under the control of a myocardial specific promoter allowed visualizing the voltage changes that occurred during the beating of the zebra fish heart *in vivo* (TSUTSUI et al. 2010). It also has been used in the mouse auditory cortex to detect electrical signaling in living mice and has been used to detect the subliminal threshold voltage responses in hippocampus neurons *in vitro* (TSUTSUI *et al.* 2013). In addition, *mer*-2 has been used during voltage-clamp and photometry in *Xenopus* oocytes (TSUTSUI *et al.* 2013). *mer,* but not *mer-2,* has been used successfully in *C. elegans* neurons (KUHARA *et al.* 2011). Therefore, the widespread previous uses of *mer* and *mer-2* suggest that it would be a good genetic tool for studying a possible voltage change during *C. elegans* fertilization.

CHAPTER IV

Methodology

Making *mermaid*-2 suitable for *C.elegans*

The *mer-2* plasmid (from Dr. Hidekazu Tsutsui, Osaka University, Japan) has a cDNA coding sequence that was not suitable for my studies because genomic DNA (containing introns) is required for high-level expression in the *C. elegans* germline (BOWMAN 2013). There are 4,699 genes known to be expressed in the *C. elegans* adult hermaphrodite germ-line, which is approximately 21% of all known *C. elegans* genes (SHA AND FIRE 2005). Therefore, Dr. Steven L'Hernault used *in vitro* DNA synthesis to create a *mer*-2 gene that has *C. elegans* codon bias and introns known from prior work to be spliced in the *C. elegans* germline (Dr. W. G. Kelly, personal communication). There was a high degree of sequence identity between the CFP and YFP parts of the original *mermaid-2* cDNA construct and we had concerns that there could be homologous recombination (either in bacteria during gene cloning or in worms during transgene formation) between these regions that might alter/delete part of the sequence. Consequently, the redundancy of the genetic code was used to select alternative codons that changed the DNA sequence within the regions encoding the two fluorescent proteins. The end result was that the degree of DNA identity between the regions encoding the two fluorescent proteins was reduced without altering the resulting protein that would be translated (made by GeneArt, Life Technology, Grand Island, NY; for sequence see Supplemental Figure 1). Restriction digestion was used by Elizabeth Gleason to create pEJG32, which has the *mer-2* gene between a *pie-1* promoter and 3'UTR that is derived from pHF10 (a plasmid from H. Furuhashi and W.G. Kelly). The pEJG32 construct should suitably drive *mer-2* expression (see Figure 2) because the *pie-1* gene is expressed during maternal loading of *C. elegans* oocytes (MERRITT *et al.*

2008).

Sequencing of *pie-1* promoter and 3' UTR junctions with gene

The plasmid was sequenced to confirm the *pie-1* promoter, *mermaid-2,* and *pie-1* 3'UTR. Plasmids were sent to UC Berkeley Sequencing Facility and the expected DNA sequence was confirmed as being present in our plasmid pEJG32.

Setup for Micoinjections

a. Pads

Pads contained 2% agarose in 10 mL of distilled water. A mixture of 2% agarose in distilled water was heated until dissolved and two drops were placed on a coverslip. After a few seconds, a second cover glass is placed on top. After about a minute the top glass was removed to reveal a penny sized, thin pad. Pads were left overnight to dry before use. However depending on weather conditions pads were also placed in a vacuum or an oven to remove extra moisture.

b. Needles

Glass needles were pulled with a Narishige PC-10 micropipette puller at heater level of 65-67. The injection solution was subject to centrifugation for five minutes before use in order to pellet any particles. The needle, when ready to inject, was loaded with 1.0 µL of injection solution. The nitrogen tank was set to approximately 15 psi. Once the needle was secured, the cover glass was positioned with worm in a drop of mineral oil on pads at a 45° angle on the injection scope. Once the worm was stuck to the pad, the gas pedal was pressed until little liquid was released but barely breaking the needle. The needle was

now open and ready to inject into the worm(s). Needles can also be etched open using hydrofluoric acid (MELLO *et al.* 1991)

Injections with *myo-2P::mcherry*

The plasmid DNA pEJG32 (*pie-1promoter::mer-2::pie-1utr*; *unc-119*) at a concentration of 50 ng/µl was co-injected with pCFJ90 (*myo-2P::mcherry*) at a concentration of 5 ng/µl into *fem-1(hc17)IV; glo-1(zu391)X* worms. The co-injection marker pCFJ90 (*myo-2P::mcherry*) makes the pharynx of the worms glow red when epi-illuminated with 596 nm light. The pCFJ90 - *Pmyo-2*::*mCherry::unc-54utr* was a gift from Erik Jorgensen (Addgene plasmid # 19327). After individual injections, each worm was placed on a plate with *E. coli* OP50 bacterial food and recovered and grown at 16°C because the *fem-1 (hc17)IV; glo-1(zu391)X* are temperature sensitive. F1s were screened for the marker after approximately 48 hours of growth. Worms were observed over three generations to confirm whether or not a line was stable.

Injections with *myo-3P::mcherry*

For these experiments, complex extra-chromosomal DNA arrays (KELLY *et al.* 1997) were created by microinjection into *fem-1(hc17)IV; glo-1(zu391)* worms. The injection mix included 50 ng/µL pEJG32 (*pie-1::mer-2::pie-1utr*), 10 ng/µL pCFJ104 (*myo-3P::mcherry::unc-54utr*) and 40 ng/µL *Pvu*I-digested N2 genomic DNA. pCFJ104 was a gift from Erik Jorgensen (Addgene plasmid # 19328) and it makes the body wall of worms glow red when they are epi-illuminated with 596 nm light. The *Pvu*I digested N2 genomic DNA helps create DNA arrays of high sequence complexity and this tends to make more stable arrays that show higher levels of gene expression (KELLY *et al.*

1997). Worms were injected individually and kept on separate plates with *E. coli* OP50 bacteria at 16°C and screened for the mCherry red fluorescent marker approximately 48 hours after microinjection.

Injections with only pEJG32

As mentioned above, the plasmid DNA pEJG32 (*pie-1::mer-2; unc-119*) includes the wild type *unc-119* gene. For these experiments, pEJG32 was injected at a concentration 100 ng/µL into *unc-119* worms. Worms were injected individually and kept on separate plates with *E. coli* OP50 bacteria at 20°C and then F1s were screened for wild-type movement (rescue of *unc-119*).

Photographing paralyzed worm

To paralyze the worms for imaging, 157.2 µL of distilled water, 40 µL of 1x sperm media at pH 7.8, 1.8 mL of dextrose, and 1 µM (50mM) levamisole solution was made. One adult hermaphrodite was picked off a plate with *OP50*, placed into levamisole buffer and photographed at 10X on an Olympus BX 60 compound microscope equipped with epi-fluorescence.

Cookworm was used to isolate genomic DNA

The presence of *mer-2* in transgenic lines was investigated by isolating total DNA from worms and subjecting it to the polymerase chain reaction (PCR) with array-specific primers. Ten adult worms were placed into a 0.2 mL PCR tube containing 10 µL of lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP40, 0.45% Tween 20, 0.01% gelatin and 1 mg/ml proteinase K), which was then briefly subjected to centrifugation. The tubes were frozen at -80^{o}, subjected to two freeze-thaw cycles and then placed in the thermocycler to run the " Cookworm Protocol" (from the Lamitina Lab

Protocols 2007 http://www.med.upenn.edu/lamitinalab/protocols.shtml): 60°C for 60 minutes, 95°C for 15 minutes, and 4°C hold. This worm lysate was used directly as the genomic DNA template in PCR.

Polymerase Chain Reaction (PCR) with Taq polymerase

The HotStar Taq polymerase kit by Qiagen (Valencia, CA) was used for all PCR reactions. All PCR reactions were performed with a denaturation temperature of 95° for 15 minutes, annealing at 55° for 30 seconds, and extension at 72° for 1 minute. PCR was repeated for 35 cycles with a final extension at 72° for 10 minutes. Primers used were AB1 (5'- AGT CGC GTC CAG TTT CGT GTC -3') and AB2 (5'- CCA TCG GCT CCG GTC TCT TG-3').

Molecular cloning for MosSCI

The enzymes *Psc*I and *Sac*II were used to restriction digest *mer-2* and pCFJ151 was digested with BssHII to create fragments that were ~9,000 base pairs and ~8,000 base pairs respectively. The fragments were gel purified using a Zymo Research kit (Irvine, CA), the ends were blunted with T4 polymerase and shrimp alkaline phosphatase (rSAP; New England Biolabs, Ipswich, MA) was used to dephosphatase the ends of vector pCFJ151. The insert will be cloned into the vector using the New England Biolabs Quick Ligation Kit and then transformed into *E. coli* DH5α cells. After plasmid DNA mini-prep, confirmation will be needed to confirm insert by using enzymes *Sac*II and *Afe*I restriction in an enzyme digest. For details about this plasmid see Figure 10.

CHAPTER V

Results

Results:

Microinjecting pEJH32 using co-injection marker *myo-2p::mcherry*

I microinjected many worms from which I identified two worms that produced progeny where the pharynx expressed mCherry. Fifty F1 candidate transgenic lines from these two Po worms were picked to separate plates and analyzed. Only one of these F1 lines produced transgenic F2 worms. The transgenic strain that contains pEJG32 (*pie-1::mer-2::pie-1utr; unc-119*) and pCFJ90 (*myo-2P::mcherry*) in *fem-1(hc17)IV; glo-1(zu391)X* hermaphrodites were imaged in the F2 generation. In addition to having a mCherry red signal in the pharynx, there is also a slight red fluorescence in the gonad, which appears to originate in the eggs. The gonad of this transgenic worm also showed green fluorescence in the vicinity of the spermatheca and vulva (Figure 6C), which is what would be expected for *mer-2* expression. There were no additional progeny with this line, as no F3s were produced. Toxicity has been observed with pCFJ90 (*myo-2P::mcherry*) (FROKJAER-JENSEN *et al.* 2008) and this is a possible reason why I could not easily get transgenic lines were not obtained when using this co-injection marker.

Microinjecting pEJG32 using co-injection marker myo-3P::mcherry

I recovered three transgenic F1 worms from one Po hermaphrodite that I injected with pCFJ104 and pEJG32. Only one of these worms passed on the *mcherry* marker to the next generation. The marker pCFJ104 (*myo-3P::mcherry*);(*fem-1 (hc17) IV; glo-1 (zu391)* hermaphrodite worm was in the F3 generation when it was photographed. Despite a clear mCherry signal in the body wall muscle, as expected for the *myo-3P* driven co-injection marker, there was not detectable green fluorescence in the gonad as

we expected should occur if *mer-2* was being expressed (Figure 7C). One possibility is that, although, I co-injected two plasmids, the *mer-2* encoding plasmid failed to incorporate in the worm transgene that formed. This possibility was investigated by PCR and the result was at least part of the *mer-2* is present in the *fem-1(hc17)IV;glo-1(zu391)* worms(Figure 9, Lane 3). This lack of detectable green fluorescence is still unexplained.

2.3 *Microinjecting pEGJ32 into unc-119 worms*

The purpose of this approach was to create transgenic worms with a single plasmid that could be identified by phenotypic rescue of *unc-119*. In theory, a transgene that rescues the *unc-119* phenotype should also contain the *mer-2* genetic information. I identified *unc-119* animals that were wild type for motility and showed that they segregated ~90% wild type, as is characteristic of extra-chromosomal array-bearing animals. This presumptive transgenic line appeared to be stable, so I characterized its properties. Worms from the F3 generation of this line do not appear to have any significant fluorescence in their germline, as I had anticipated would be the case if *mer-2* was expressed properly. Their oocytes were imaged since *mer-2* should be hypothetically expressed in the oocytes (Figure 8C.). However, the transgenic strain has features that, while not fully explained, suggest that the *mer-2* sequence might be present. Specifically, when placed at 25°C, transgenic hermaphrodites are sterile, but at 16°C and 20°C the majority were able to produce progeny. Prior work has not shown any effect of *C. elegans* wild-type *unc-119* expression on *C. elegans* self-fertility and *unc-119* has been used as a common marker in micro-bombardment and microinjections (MADURO AND PILGRIM 1995); this suggests that this effect might be

related to the presence of *mer-2.* The temperature sensitive self-sterile hermaphrodites were cross fertile when mated to *him-5* males. This indicates that the temperature sensitive sterility was due to defective sperm.

2.4 **Molecular cloning for MosSCI**

No colonies appeared on the agar plate after transformation and therefore the *mer-2* was not inserted into the pCFJ151 vector.

CHAPTER VI

Discussion

Discussion:

The purpose of my project was to create transgenic lines expressing an *in vivo* voltage sensor, *mer-2*, in *C. elegans* hermaphrodites. I expected to find a fluorescent green signal regardless of whether or not there was a voltage change during fertilization. I got encouraging first signs that this strategy can work by examining worms prior to transgene stabilization. However, in presumptive stabilized lines, what I found was a dim signal and it is unclear if this will be usable.

MER-2 and similar proteins cause a voltage-independent green signal and this signal has been observed in a variety of species including frogs, zebrafish, mice, and in *C. elegans* neuron cells (TSUTSUI *et al.* 2008; 2010; 2013; KUHARA *et al.* 2011). Consequently, I anticipated that if the transgenes I created were expressed in the correct tissue-specific fashion, we would observe a MER-2-derived green signal in the hermaphrodite germline. One potentially confounding factor is that the *C. elegans* gut has a considerable amount of autofluorescence, so that a dim germline-derived green signal might be overwhelmed by the gut-derived background fluorescence (KLASS 1977). For this reason, *fem-1 (hc17) IV; glo-1 (zu391*) worms were chosen for injection because *glo-1* mutants lack gut auto-fluorescence so one avoids any background fluorescence during imaging. When injecting the *unc-119* worms, the pEJG32 plasmid includes *unc-119*, so no co-injection marker was needed because transgenic animals will be wild type for motility.

Many assumptions were made when the *mer-2* cDNA was analyzed and then converted into a *C. elegans* gene by Dr. L'Hernault. One potential concern with using the *mer-2* construct created in our lab is that we did not know if it is capable of

functioning in the *C. elegans* germline. In one experiment, a transgenic strain (pEJG32 and pCFJ90 in *fem-1(hc17) IV; glo-1(zu391)X* worms; Figure 6C), shows a pronounced green signal in the spermatheca and vulva This suggests the *mer-2* gene we created *in vitro,* making assumptions about codon bias and introns, can be expressed in the *C. elegans* germ-line.

It has been seen previously in my laboratory that *gfp or mcherry* driven by *pie-1* can be toxic to the worms at high concentrations and also noted by both the Fire (Jamie Fleenor, Lisa Timmons, SiQun Xu, Kelly Liu, Bill Kelly and Andrew Fire, unpublished observations) and Jorgensen labs (FROKJAER-JENSEN *et al.* 2008). Furthermore, the transgene failed to transmit to any viable worms in the F3 generation, which is a phenomenon predicted to result from *myo-2P::mcherry* associated toxicity. Additionally, some of the worms that had the *myo-2* co-injection marker showed an unusually large pharynx and slow, sickly growth with growth arresting at an L1-L2 stage which makes sense because it is at L1 when worms first need to use their pharynx to feed and grow.

When analyzing the *myo-3* co-injection marker worms, I used PCR to confirm that the *mer-2* construct was, in fact, present in the transgene. It is possible the worm incorporated the visible marker, but not the *mer-2*, into the transgene. In my opinion, this is highly unlikely as there is a higher concentration of *mer-2* than co-marker in injection solution. Nonetheless, if this proves to be a consistent feature of multiple transgenes, it would suggest that the *mer-2* construct created by Dr. L'Hernault is somehow toxic.

After PCR, the pEJG32 with *mer-2* was confirmed in *fem-1(hc17)IV; glo-1(zu391)* worms by primers AB1/AB2 (Figure 9) . The PCR did not work in the *unc-119* transgenic

line despite the fact the plasmid, pEJG32, contains both the *unc-119* and *mer-2* genes. I cannot conclude it is absent based on a negative PCR. If time permitted I would have optimized PCR and confirmed *unc-119* is, in fact, in the extra-chromosomal array.

Characterizing *C. elegans* transgenic strains is associated with a variety of challenges. First of all, the extra-chromosomal arrays are lost at some level, and this can make analysis difficult. Transmission of extra-chromosomal arrays is dependent on array size and ranges from 10 to 90% from generation to generation (MELLO AND FIRE 1995). Unlike somatic cells in *C. elegans*, germ cells are known to be efficient in silencing genes that are present at multi-copies (ROBERT *et al.* 2004). It is estimated that the extra-chromosomal arrays have at least 80-300 copies of the injected plasmids (STINCHCOMB *et al.* 1985); (FIRE AND WATERSTON 1989).

Some transgenes with a high number of gene copies can exhibit over expression (KELLY *et al.* 1997). This is one of the reasons why N2 DNA was added to the injection mixture during the *myo-3* injection protocol creating a complex extra-chromosomal array. However, this method is known to result in a low frequency of extra-chromosomal arrays relative to the number of required microinjections, so it is not a popular method (MELLO *et al.* 1991).

For my *unc-119* injection experiments, I lowered the concentration of *mer-2* from 50 ng/μl to 10 ng/μl because I became worried that *mer-2* might be toxic. However, this does not always work because silencing or over expression can still occur, due to tandem repeated sequences in the array (OKKEMA *et al.* 1993; KELLY *et al.* 1997). Another concern regarding the *C. elegans* germline is that 3'UTRs usually specify expression, unlike in somatic cells where promoters do most of the gene expression

regulation (MERRITT *et al.* 2008). With the germ-line complexity, and the potential drawbacks of injections, many variables could affect why the *mer-2* is not being detectably expressed.

It is possible to use another method instead of extra-chromosomal array microinjections to insert foreign DNA into *C. elegans*. This method is called MosSCI and was discussed briefly in the Introduction. It has been seen that when injecting extra-chromosomal arrays, the extra-chromosomal array may not be expressed at 100% transmittance unless the extra-chromosomal array is integrated into the chromosome, like in the case of MosSCI (FROKJAER-JENSEN *et al.* 2008). This method lowers the chance of silencing or over expression since the gene is located on a pre-selected site on a chromosome, is present as a single copy and does not disrupt any essential gene or genes.

CHAPTER VII

Next Steps

Next Steps:

If the *mer-2* were present in transgenic worms, we would expect to see a green fluorescent signal indicating expression. However, no signal was seen. PCR confirmed the pEJG32 with *mer-2* was, in fact, in the *fem-1(hc17)IV; glo-1(zu391)* transgenic worms. The PCR did not yield a positive result in rescued *unc-119* transgenic line, despite the fact that the plasmid, pEJG32, contained both the *unc-119* and *mer-2* genes. I cannot conclude that the plasmid is absent based on a negative PCR, but if time permitted, I would continue to optimize PCR and confirm *unc-119* is in fact in the extra-chromosomal array, as seems likely.

The presence of a fluorescent signal in oocytes is an unambiguous indicator that the *mer-2*-encoding plasmid is functioning properly. I have some hints that this is the case, but I do not yet have the sort of bright, stable fluorescence that will allow analysis of fertilization-defective mutants. As mentioned previously, the *C. elegans* gut has a considerable amount of autofluorescence, so it is possible that a dim germline-derived green signal might be overwhelmed by the gut-derived background fluorescence (KLASS 1977). RT-PCR is much more sensitive than fluorescence and would allow me to determine if my *fem-1(hc17)IV; glo-1(zu391)X mer-2* transgenic line transcribes *mer-2* mRNA . Should my line prove to transcribe *mer-2* mRNA, this would mean that epigenetic silencing is not a problem and we would have to look elsewhere for a solution. For instance, we may need to manipulate physiological conditions, such as pH, to see if it is possible to achieve a detectable fluorescent signal.

Although the PCR did not confirm the presence of the *mer-2* in the *unc-119* rescued worms, the gene should be there as the worms show wild-type movement. A

negative PCR result is equivocal, so the next steps will be to confirm that *unc-119* is in the extra-chromosomal array, as well as continuing to optimize the PCR conditions. One unexpected result was that my rescued *unc-119* worms were temperature-sensitive sterile at 25°C and at least partially sterile at 20°C. This sterility is sperm-based because transgenic worms are cross-fertile after mating to wild type males.

The end goal is to obtain a *mer-2* transgenic line with the transgene integrated into a chromosome using MosSCI. This would be done by cloning *pie-1P::mer-2::pie-1utr* into pCFJ151, which is the vector used to generate MosSCI insertion at the ttTi5605 location on chromosome II (FROKJAER-JENSEN *et al.* 2008). This should result in a low copy number, chromosomally integrated *mer-2* gene that has an excellent chance of escaping epigenetic silencing and other phenomena that prevent high-level expression in the *C. elegans* germline.

CHAPTER VIII

Big Picture

Big picture:

Studying *C. elegans* mutants can provide insight regarding the components needed for sperm-egg interactions during fertilization (SINGSON 2001). The *mermaid-2* voltage sensor is a possible way to utilize a genetic tool *in vivo* to study fertilization. It may help in further classifying *spe-9* class mutants and other fertilization mutants.

These *spe-9* class mutants are able to make contact with an oocyte, but not complete fertilization despite having sperm that are same in motility and cytology as wild-type (SINGSON *et al.* 1998). This is especially important since several of the *spe* genes are possibly orthologues of mammal genes (NISHIMURA AND L'HERNAULT 2010). This suggests that *C. elegans* and mammals may share common mechanisms during male germ-line functions. Fertilization in *C. elegans* involves hundreds of sperm all trying to fertilize a single oocyte in the confined space of the spermatheca, yet polyspermy is not observed. The phenomena surrounding *C. elegans* fertilization strongly suggest that a fast block to polyspermy exists in this species and that the *mer-2* voltage sensor would be a useful tool for its analysis.

Figures:

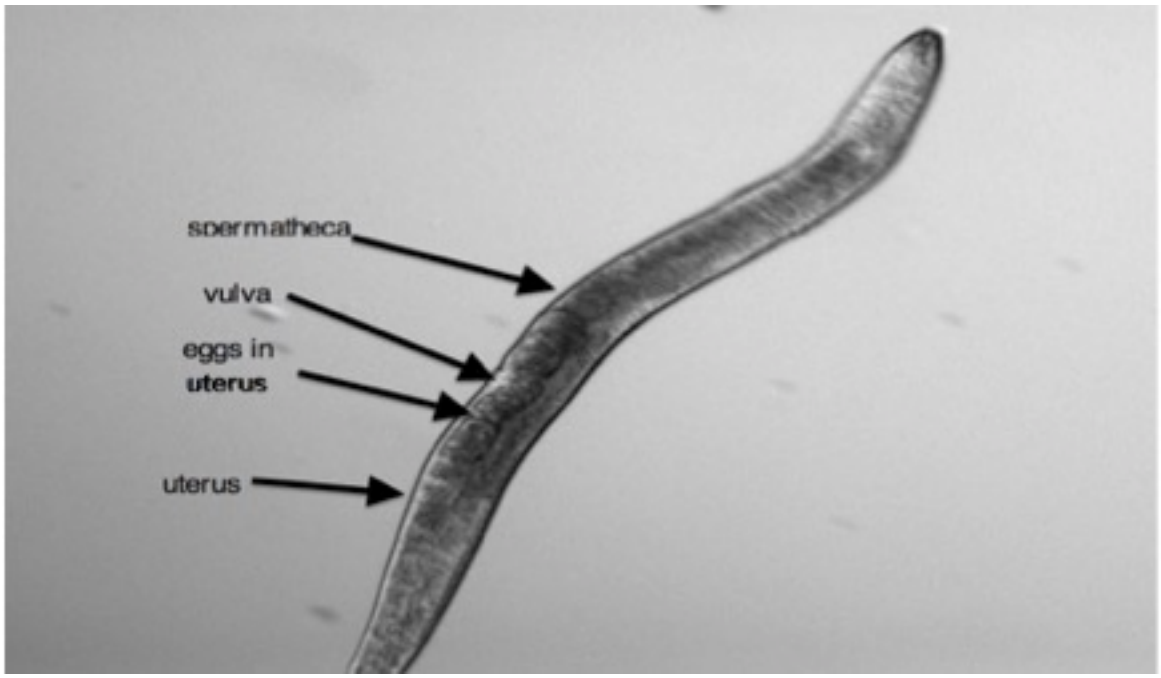


Figure 1: The reproductive anatomy of a *C. elegans* hermaphrodite worm.

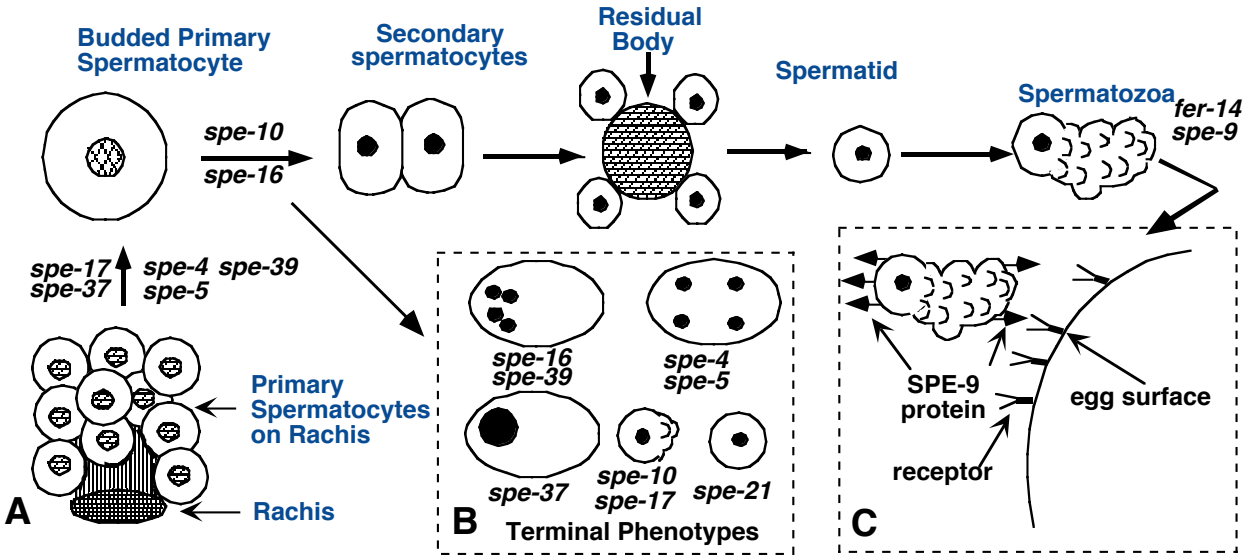


Figure 2: Basic overview of spermatogenesis and fertilization in *C. elegans*. The *spe-9* class (genotype of interest) includes: *spe-9, spe-13, fer-14, spe-36, spe-38, spe-41,izumo* and *spe-42.* Picture retrieved from: L'Hernault, S. W. "Spermatogenesis." Worm Book. http://www.wormbook.org/chapters/www_spermatogenesis/spermatogenesis.html

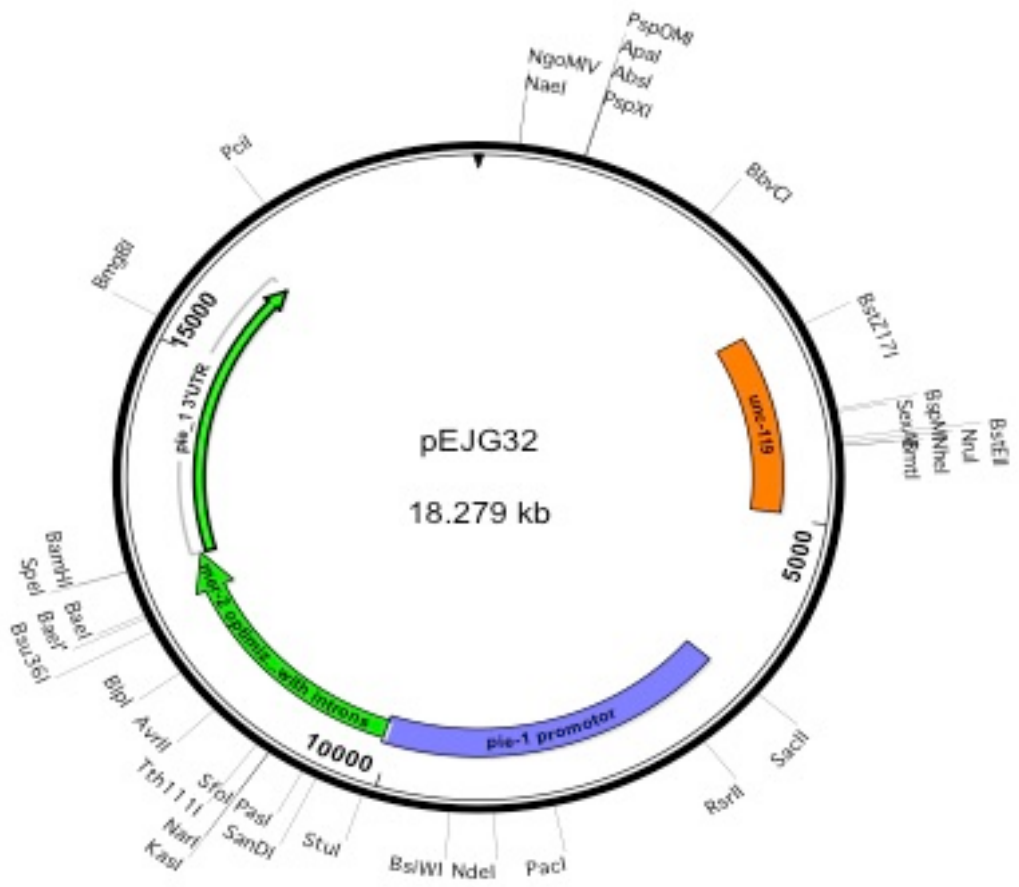


Figure 3: The pEJG32 *mermaid-2 (mer-2*) encoding plasmid is ~18.3 kb long, with restriction sites shown. The plasmid is derived from pHF10 (H. Furuhashi and W. Kelly, unpublished) and it has a *pie-1* promoter driving the *mer-2* sequence that is followed by the *pie-1* 3'UTR. This plasmid also has the wild type *C. elegans unc-119* gene, which is known to allow rescue of *unc-119* loss of function mutants.

Figure 4: The three dimensional structure of the *Ciona intestinalis* phosphatase (Ci-VSP) protein is exquisitely sensitive to cellular voltage potential and at negative voltage potential, *mer-2* expression results in a 483 nm cyan signal when a cell is illuminated with 438 nm (violet) light. (CFP, Cyan Fluorescent protein; YFP, Yellow Fluorescent Protein)

Figure 5: When the membrane potential increases the tripartite protein folds so as to bring the cyan and yellow coral fluorescent proteins in close proximity. This close proximity allows fluorescence resonance energy transfer (FRET) between the cyan donor and the 547 nm yellow acceptor, resulting in a yellow signal. (CFP, Cyan Fluorescent protein; YFP, Yellow Fluorescent Protein)

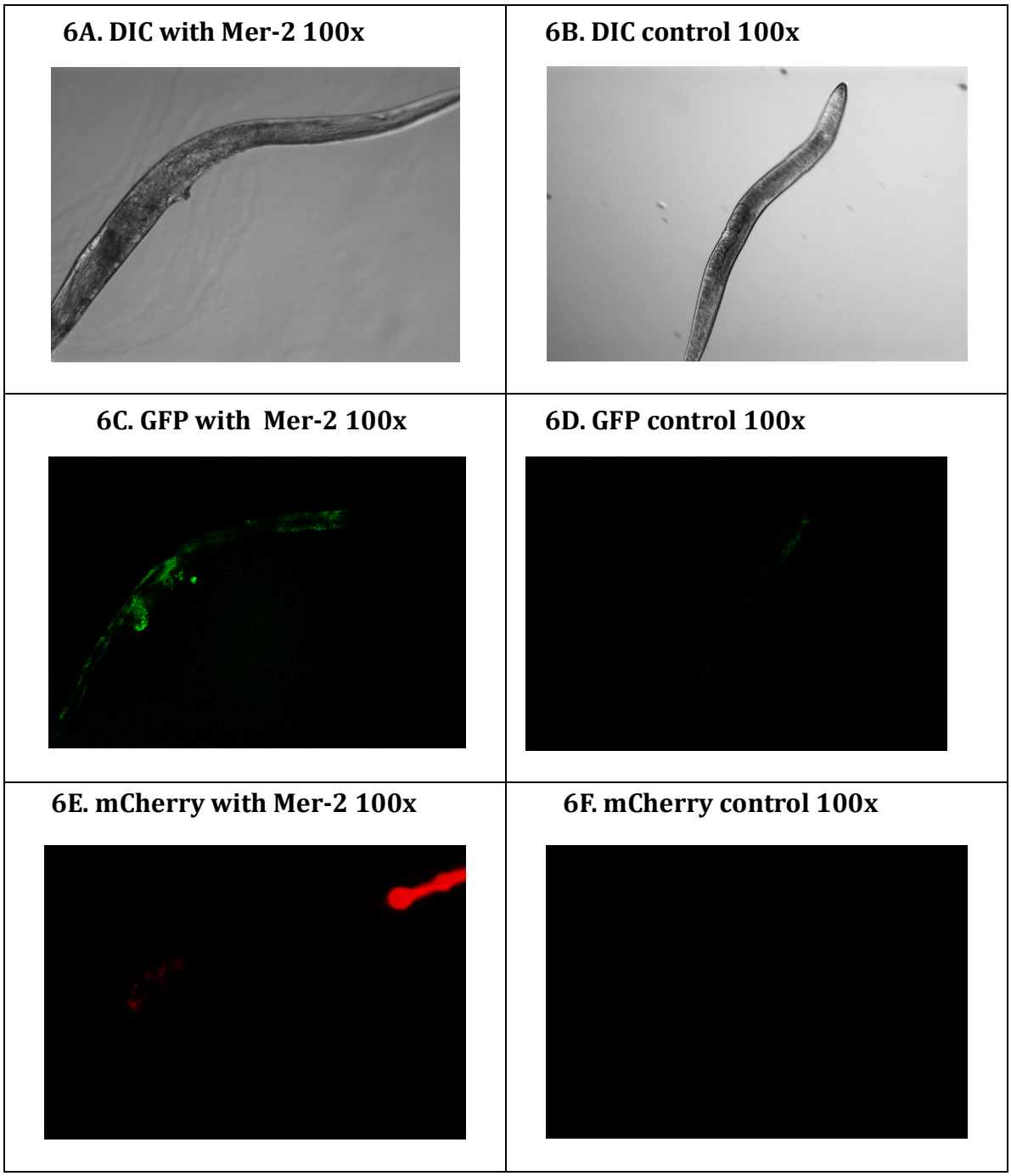


Figure 6: pEJG32 (*pie-1::mer-2::pie-1utr*) and pCFJ90 (*myo-2P::mcherry*) were co-injected into the *fem-1(hc17)IV; glo-1(zu391)* worms. The images are at 100X and show DIC, GFP, and mCherry channels. The control (B, D, F) is a *fem-1 (hc17)IV; glo-1(zu391)* nontransgenic worm that does not express any fluorescent proteins.

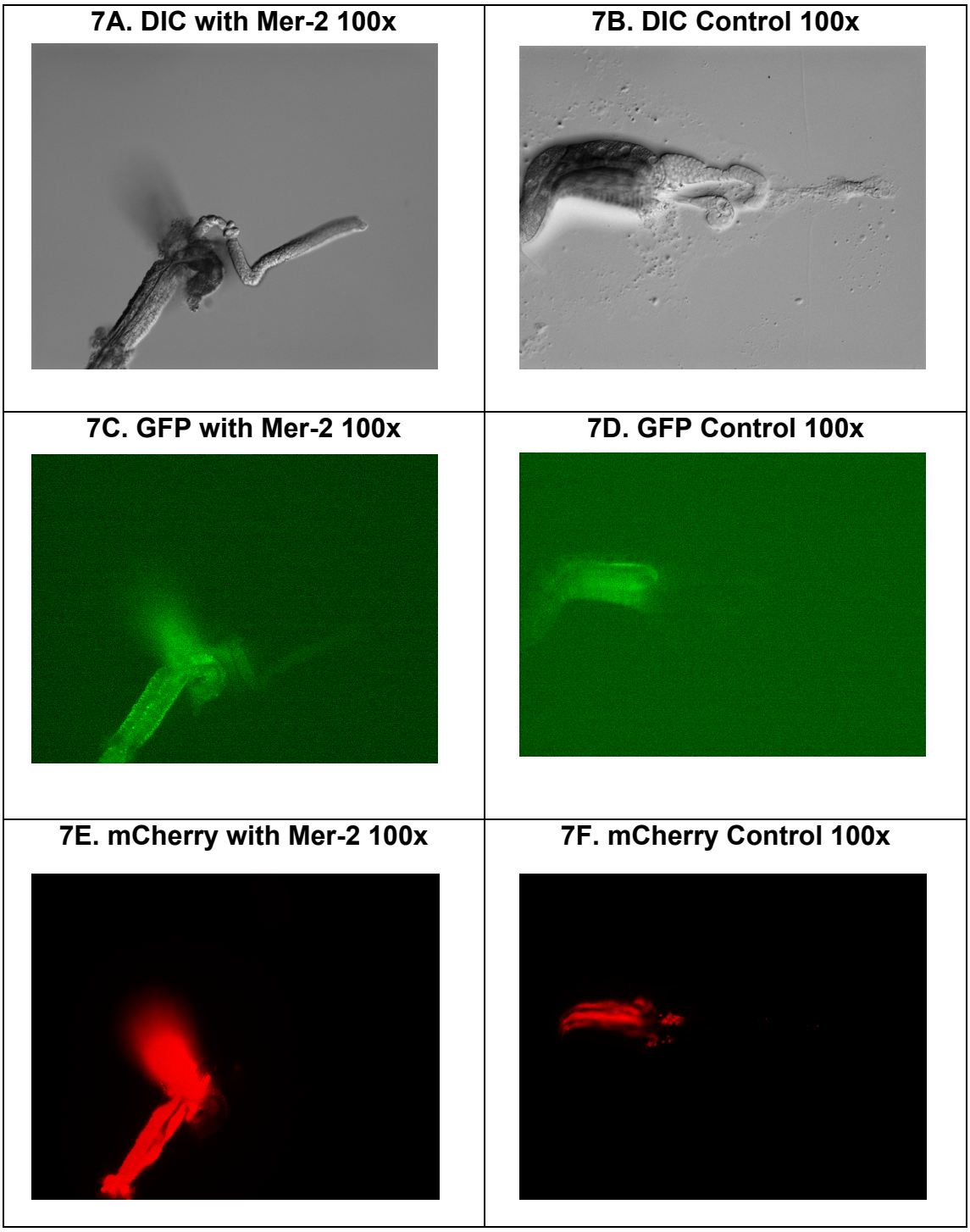


Figure 7: pEJG32 (*pie-1::mer-2::pie-1utr*) co-injected with pCFJ104 (*myo-3::mcherry*) into *fem-1(hc17)IV; glo-1(zu391)* worms (7A, C and E). Images taken at 100X in the DIC, GFP, and mCherry channels. There is no significant difference in the GFP signal seen in 7C as compared to the control *ebEX570* (*myo-3::gfp*) in 7D.

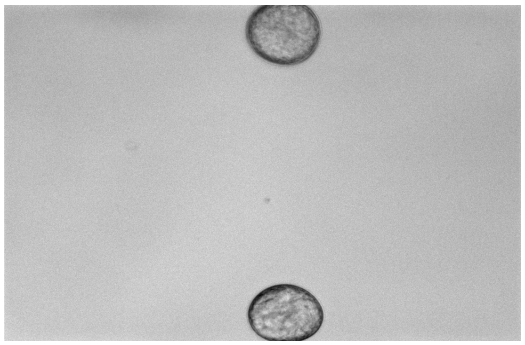
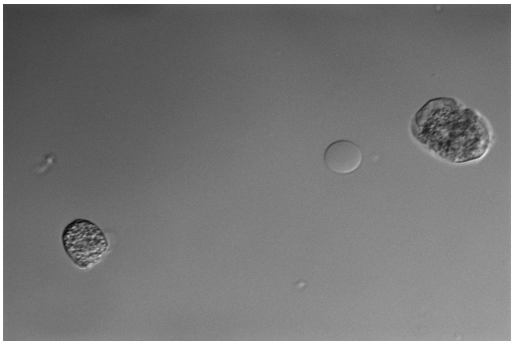
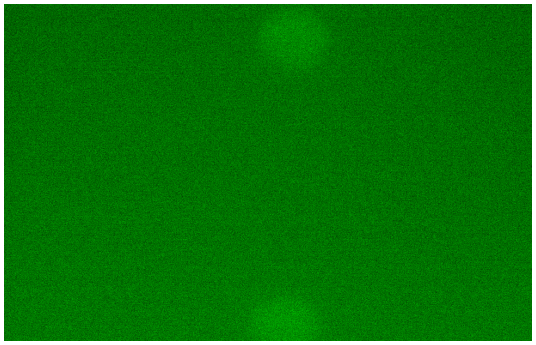
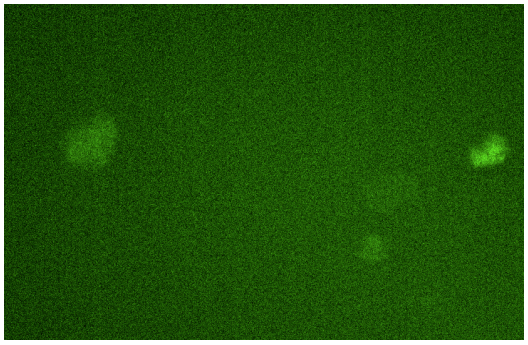
8A. DIC oocytes in *unc-119* injected *worms* with *mer-2* 400x	8B. DIC oocytes control 400x
	
8C. GFP oocytes in *unc-119* injected worms with *mer*-2 400x	**8D. GFP oocytes control 400x**
	

Figure 8: Oocytes from a potential pEJG32 (*pie-1::mer-2::pie-1utr)* transgenic strain in *unc-119* worms. Worms were dissected in sperm media (pH7.8) and viewed at 400X in the DIC, GFP, and TR channels. There was no significant difference in the GFP channel when oocytes from the potential transgenic line (8C) were compared to oocytes from the *fer-1* nontransgenic control (8D).

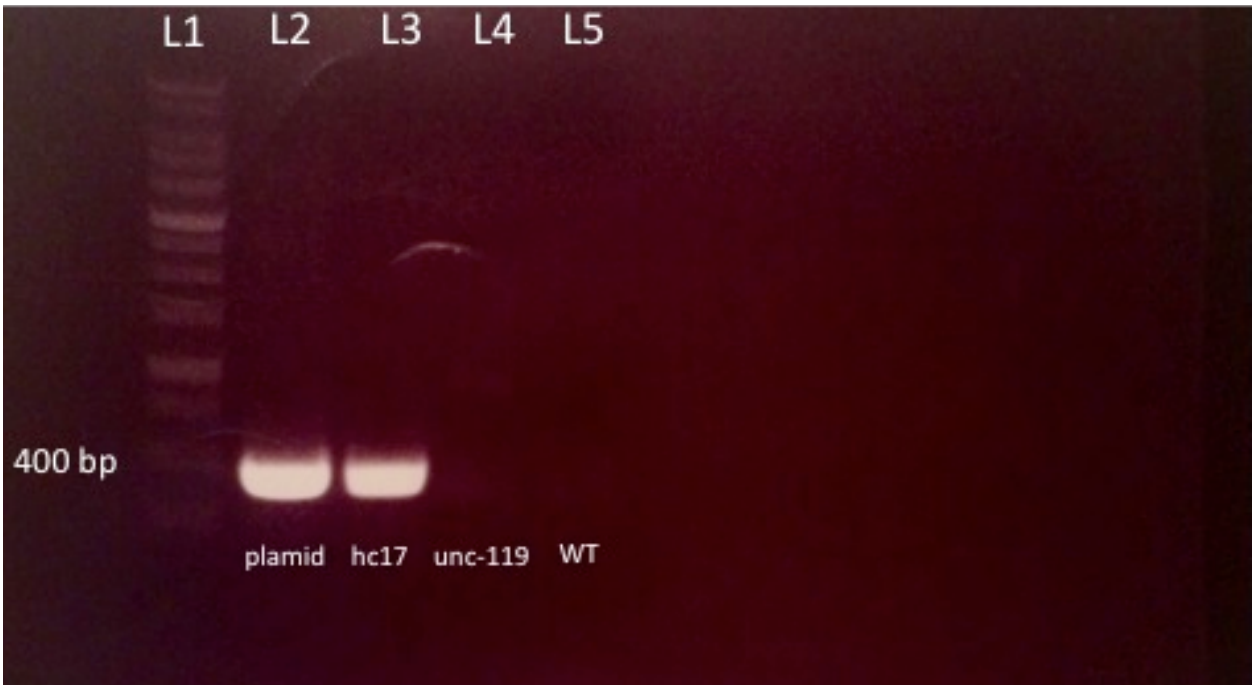


Figure 9: In lane 1 are size standards and in lane three are the PCR product derived from *fem-1(hc17) IV ;glo-1(zu391)* worms that had been injected with both pEJG32 (*pie-1::mer-2::pie-1*) and pCFJ104(*myo-3::mcherry*). The ~400 base pair band that amplified with primer pairs AB1/AB2 confirms the *mer-2* is present in the worms. In lane 2 is the purified *mer-2* plasmid, which is the positive control. In lane 4 and in lane 5 are the PCR products from *unc-119* worms injected with pEJG32 and wild type (with no transgene), respectively, and in neither case were bands present. There should have been a *mer-2* derived PCR product in lane 4. As expected, no bands were visible in the wild-type negative control.

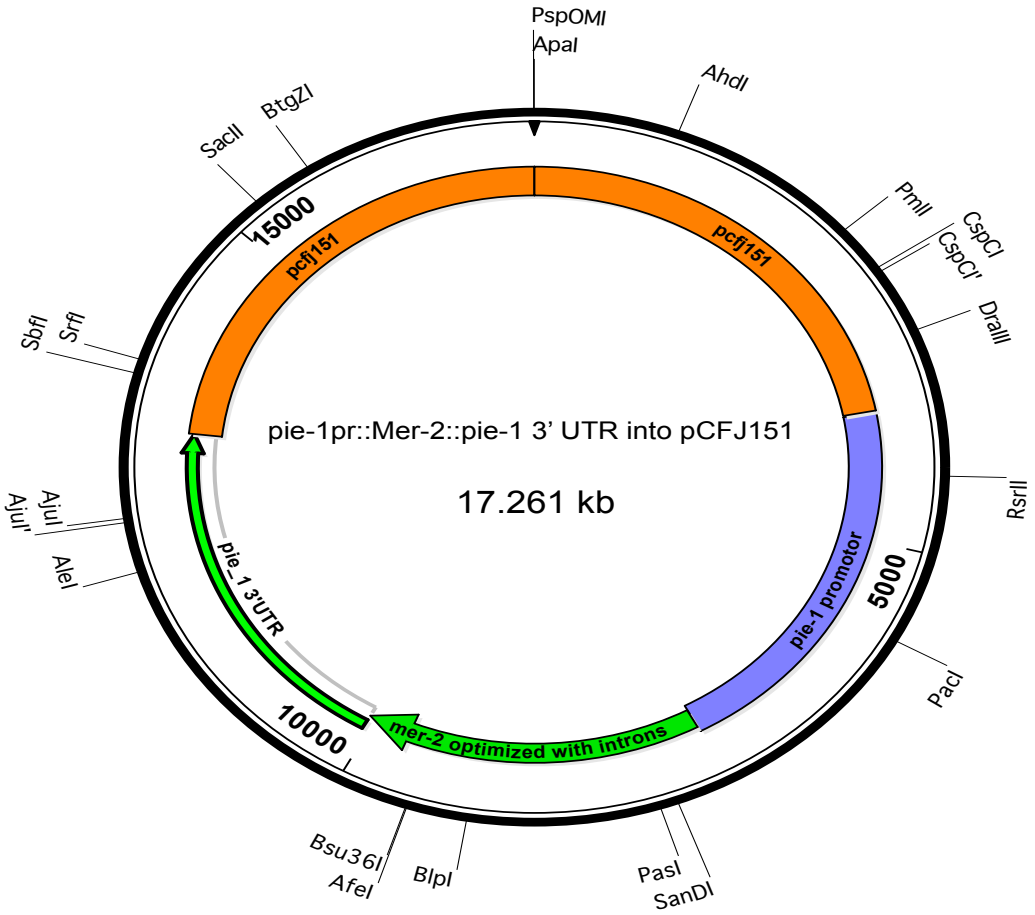


Figure 10:The *pie-1p::mer-2::pie-1* 3' UTR into pCFJ151 proposal plasmid that will be used for MosSCI insertion at the ttTi5605 location on chromosome II.

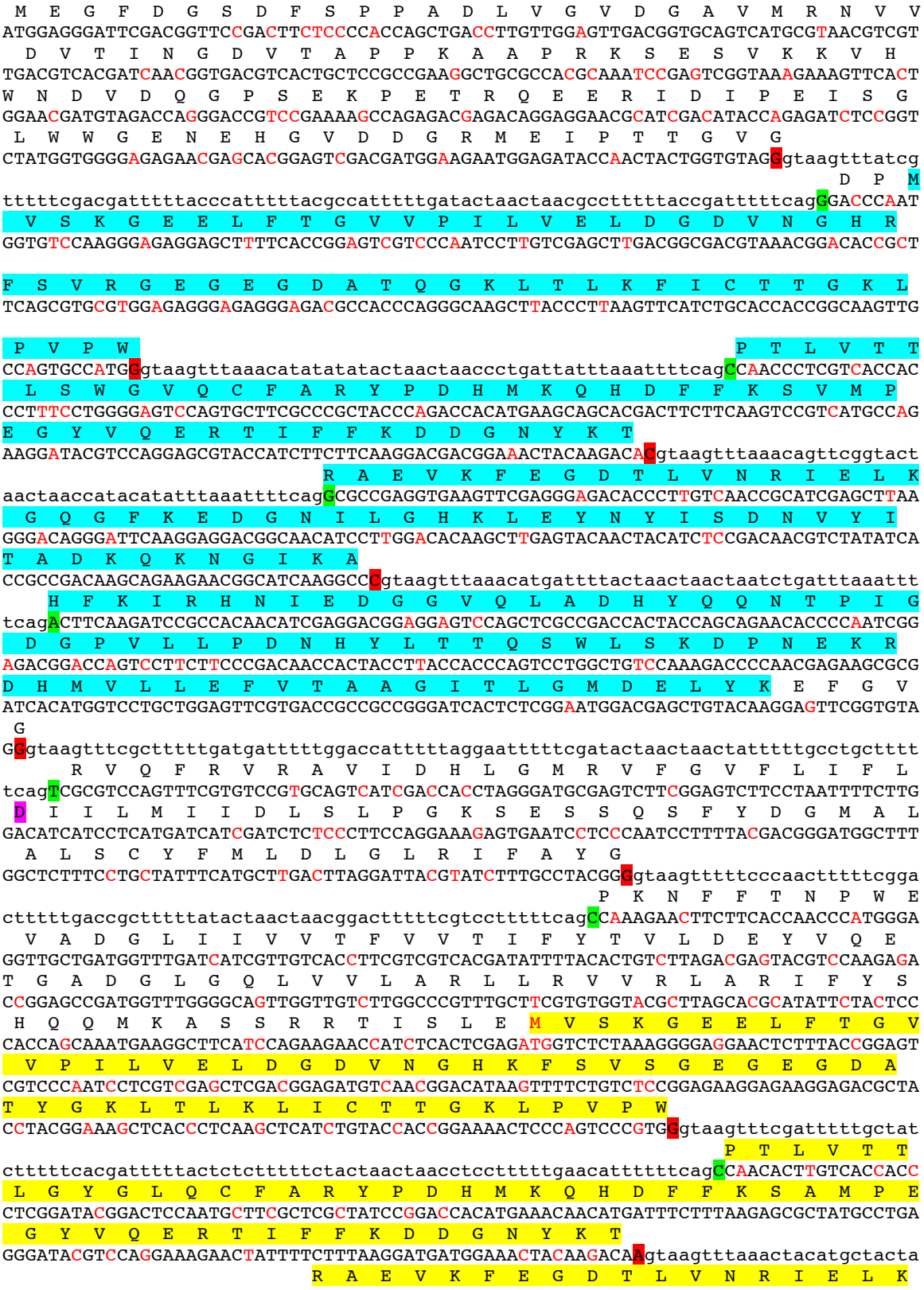
Supplemental Table 1:

Name of *spe*-9 class mutant	Predicted protein	Reference
spe-9	Sperm specific transmembrane protein	(L'HERNAULT *et al.* 1988)
spe-13	Still being investigated	(L'HERNAULT *et al.* 1988; PUTIRI *et al.* 2004)
spe-36	Still being investigated	(L'HERNAULT *et al.* 1988; L'HERNAULT 2006)
spe-38	4-Pass transmembrane protein	(CHATTERJEE *et al.* 2005)
spe-41	Calcium-permeable cation channel	(XU AND STERNBERG 2003)
spe-42	transmembrane protein with the DC-STAMP and RING finger domains	(KROFT *et al.* 2005)
izumo	protein translocates to the plasma membrane during sperm activation	(XU AND STERNBERG 2003; SINGARAVELU *et al.* 2012)
fer-14	Transmembrane protein	KROFT *et al.*, unpublished

All of the known *spe-9* class mutants produce sperm with cytology that is indistinguishable from that of wild type sperm. However, these mutant sperm are unable to complete fertilization even when they contact an oocyte.

Supplemental Figure 1:

mermaid-2: a codon scrambled and optimized *C. elegans* gene



Supplemental Figure 1 legend: The *C. elegans mermaid2* gene was created by Dr. S. W. L'Hernault and *in vitro* synthesized. While the amino acid sequence matches the previously published sequence (Tsutsui et al., 2013), redundant codons have been used to change the DNA homology between the cyan and yellow fluorescent protein encoding sequences. The codon changes to the DNA sequence are indicated by either red or green highlighting. An aspartate amino acid residue (D) is within the *Ciona*-derived phosphatase voltage sensor encoding sequence, and this residue is changed to an arginine in a control sequence (not shown) to make a voltage insensitive null mutation.

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