Enhancing sustainability of shellfish aquaculture through streamlined maturation control

Final Report

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1 Project Rationale

Sterile shellfish are both a market driven need and an ecologically sustainable approach to increase food production via aquaculture. Sterility has clear advantages in shellfish aquaculture including the ability to 1) increase growth and flesh quality as nutrient/energy reserves normally utilized in gonad development are directed to somatic tissue, and 2) preclude genetic contamination of wild, native bivalve populations by farmed conspecifics. To increase the productivity and sustainability of the shellfish aquaculture sector, while at the same time enabling hatchery responsiveness to both environmental challenges and market demands through breeding and maturation control, a time-efficient, practical, and cost-effective means to produce sterile shellfish is critically needed. The current method for reproductive impairment involves producing triploid oysters, which are effectively sterile. However, generating triploid oysters from a selected line of oysters can take up to 10 years and is not 100% effective. An attractive alternative to ploidy manipulation, that very recently has been shown to be successful in finfish aquaculture, is the induction of sterility by inactivation of genes essential for germ cell formation and development. It is the germ cell line that ultimately develops into gametes, therefore disrupting their formation and development blocks any possibility of fertility. A thorough understanding of genes involved in germ cell fate is a critical first step toward controlling reproduction through molecular approaches such as these. Given recent advances in finfishes, the shellfish industry is now in an ideal position to adapt and adopt these technological advances. Development of this technology in bivalve shellfish requires knowledge of the specific genes essential for bivalve germ cell fate. Our proposal to advance this gap in knowledge is pivotal to achieving a thriving, environmentally-sustainable shellfish industry. The specific objectives of the project are to a) Characterize genomic processes involved in germ cell specification in Pacific oysters and b) Optimize delivery techniques of custom molecules to oyster embryos.

2 Characterize genomic processes involved in germ cell specification in Pacific oysters

2.1 Background

Primordial germ cells (PGCs) are essential for the development of reproductive cells like eggs and sperm. They form early in an embryo's development and eventually give rise to germinal stem cells. There are different ways that PGCs are specified in organisms, which can lead to varying reproductive mechanisms. Understanding how PGCs work is important for managing reproduction in the aquaculture industry, especially in producing sterile organisms.

In bivalves, such as oysters, the study of PGCs is still in its early stages. Researchers have identified a gene called vasa, which is important for PGC development in these animals. However, more research is needed to understand other genes involved in this process.

Studying PGCs has been challenging due to the difficulty of isolating these cells from other cell types in embryos. Recent advancements in single-cell RNA sequencing technology offer a new approach to studying PGCs by allowing researchers to analyze individual cells. This method is particularly useful for studying PGCs in bivalves, as they make up only a small number of cells in developing embryos. By applying this technology to early Pacific oyster embryos, we hope to gain a better understanding of the genes involved in PGC development in bivalves.

2.2 Research Activity

2.2.1 Methods

The study involves two sampling periods to identify and characterize genes uniquely expressed in these cells present during early development. The first experiment focuses on the earliest developmental stages, from the first cell division through the blastula stage. These stages were chosen as previous research has shown that the germ line marker, vasa, is restricted to a small number of cells during these stages. The second experiment targets the gastrula stage embryos, which represent a more differentiated embryonic stage, but still containing a small enough number of cells per embryo to sequence representative germ cells.

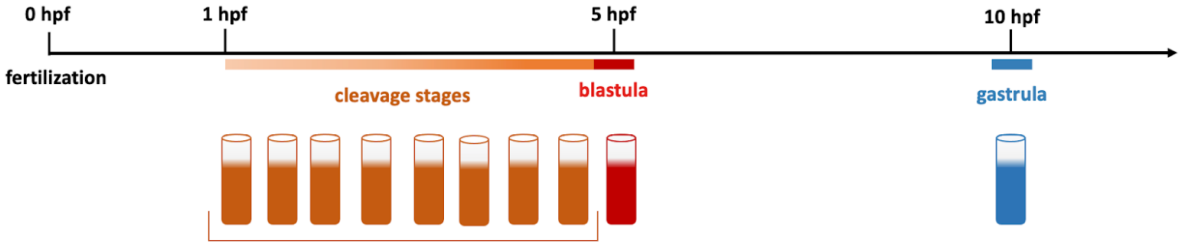

Figure 1: Schematic representation of sampling design. The first experiment, referred to as "Cl/Bla" throughout, represents continuous stages of development from cleavage stage to blastula embryos (i.e. all samples through blastula collected as consistent time intervals). Cleavage stage embryos (in orange) were pooled into a single sample representing all cleavage stages. A sample of blastula stage embryos (red) were collected and sequenced in a separate library to anchor developmental time, but cells from this experiment are analyzed together. A second experiment was performed where embryos representing gastrula stage embryos were sampled and analyzed separately.

Researchers collected samples of embryos from various developmental stages, including cleavage, blastula, and gastrula stages. They prepared two batches of embryos for cell dissociation and library preparation. The first batch represented a pool of the first eight samples, while the second batch represented the blastula stage. In a separate experiment, gastrula stage embryos were collected and prepared for cell dissociation.

Cells were dissociated using enzymatic digestion and prepared for sequencing using the Chromium platform. The resulting single-cell mRNA libraries were sequenced, and the data was processed using Cell Ranger software. UMAP (Uniform Manifold Approximation and Projection) was used for visualization and clustering of the cells, with marker gene identification performed using the Monocle3 software. Lastly, an iterative BLAST comparison was used for comparative annotation of the C. gigas gene set with model invertebrates, such as C. elegans, D. melanogaster, and S. purpuratus.

2.2.2 Results

Scientists conducted a study to better understand the development of reproductive cells in Pacific oysters. They focused on the role of a gene called vasa, which is known to be important for the development of primordial germ cells (PGCs), the cells that eventually give rise to eggs and sperm.

Using advanced sequencing techniques, researchers analyzed thousands of cells from early-stage oyster embryos. They were able to identify different groups of cells based on their gene expression patterns. Among these groups, one cluster of cells showed high levels of vasa expression, suggesting they were likely to be PGCs or their precursors.

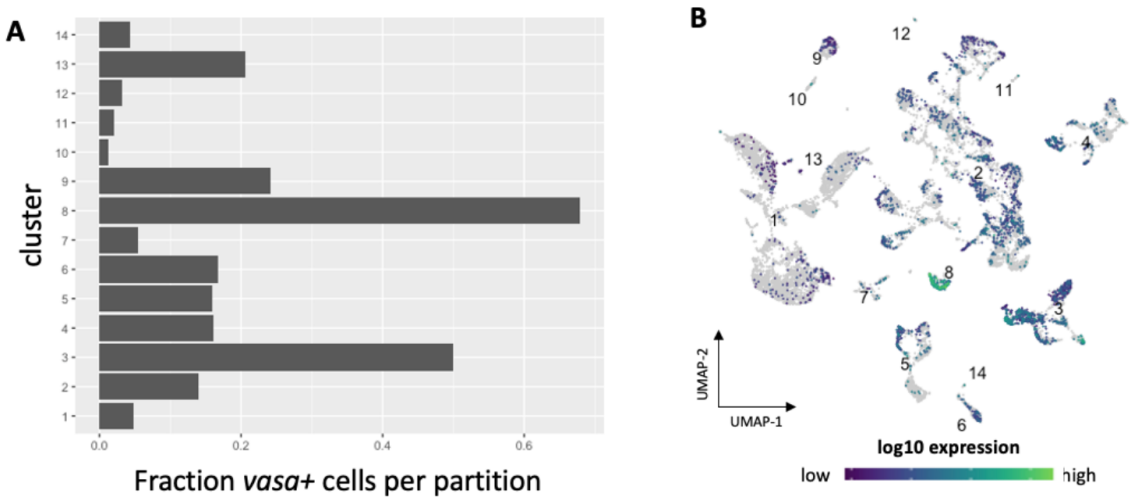

Figure 2: **vasa positive cells in C. gigas gastrula.** (A) Proportion of vasa positive cells per cluster in the gastrula cells. (B) UMAP of gastrula stage cells where color represents vasa (LOC105335166) expression (log10).

In addition to vasa, the study also found other genes that were specifically expressed in these putative PGCs, including a conserved germline marker called nanos. The identification of these markers could help scientists better understand the processes that govern reproductive cell development in oysters and other related species.

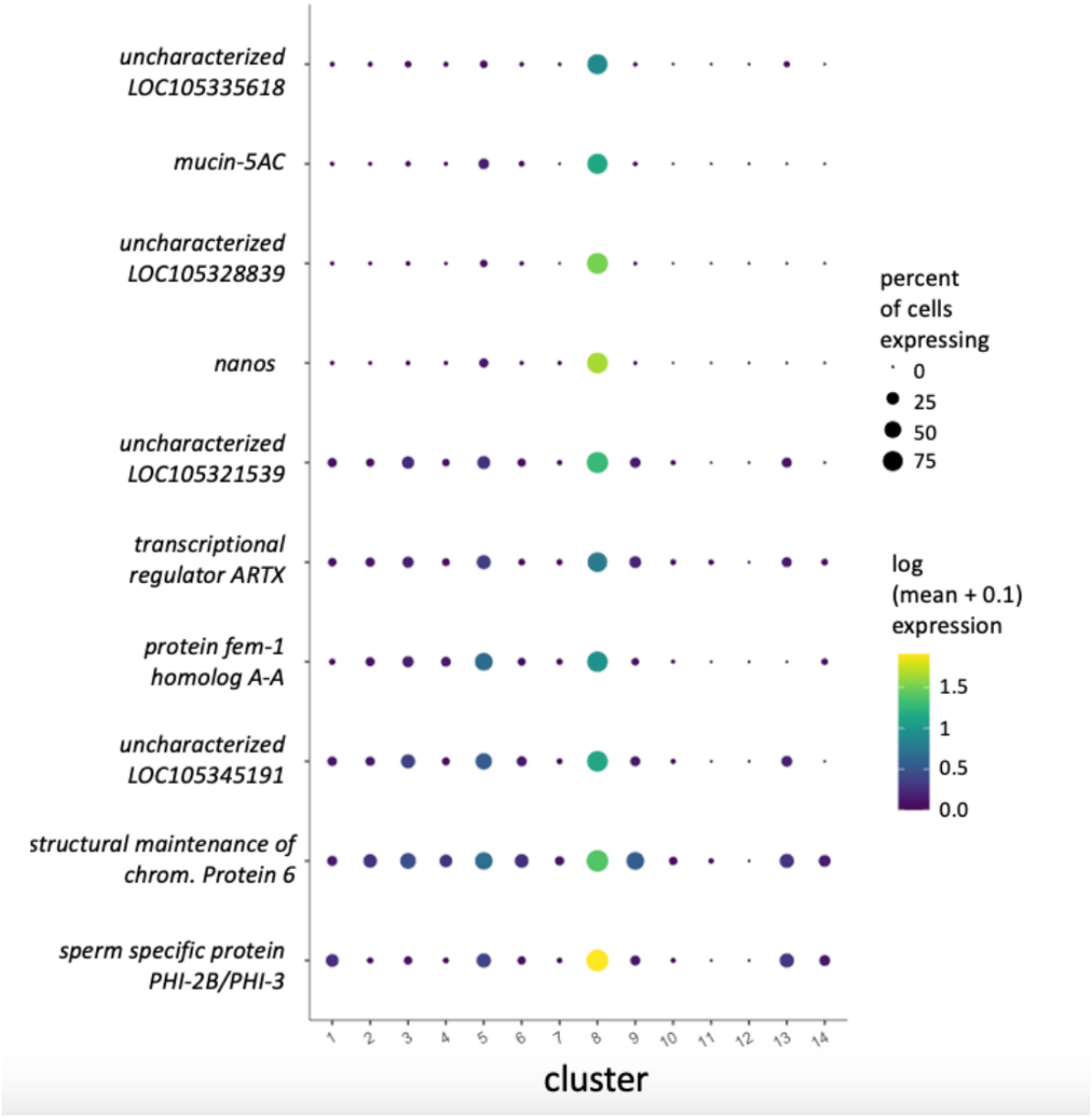

Figure 3: Marker genes associated with putative PGC in Pacific oysters. Expression characterization of select pPGC marker genes across 14 clusters of gastrula cells. The percent number of cells expressing a gene is shown by circle size, with color indicating expression level (log (mean + 0.1).

Besides focusing on PGCs, researchers also identified other cell types in the developing embryos, such as ciliary cells, muscle cells, and immune cells. This comprehensive analysis of gene expression in early-stage oyster embryos could provide valuable insights into the complex processes that govern the development of these organisms.

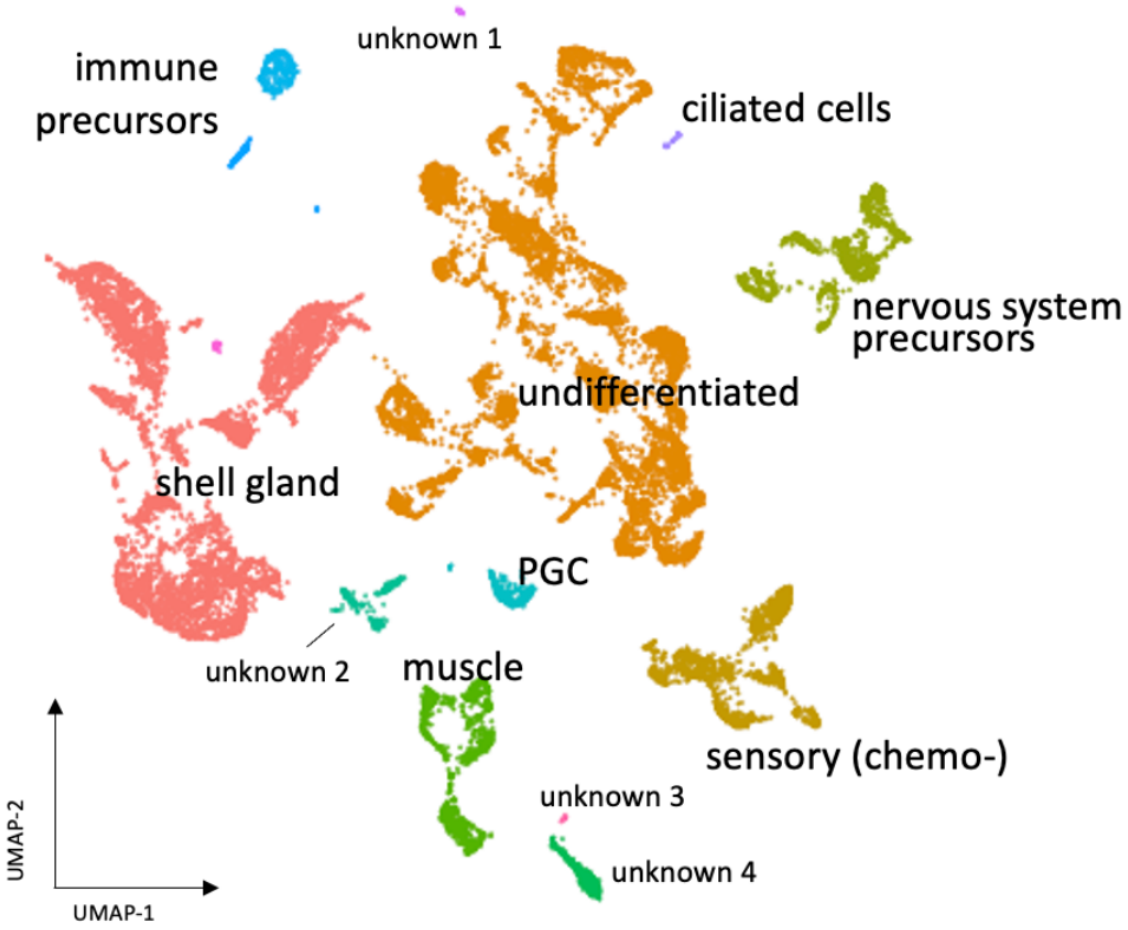

Figure 4: Multi-state expression atlas of gastrula cells. UMAP of gastrula cell states where colors correspond to clusters and cell states are annotated according to marker genes function.

2.2.3 Highlights

The three most important outcomes of these results are:

1. Identification of putative primordial germ cells (PGCs): The study identified a cluster of cells with high levels of vasa expression, suggesting they are likely PGCs or their precursors. This finding is crucial for understanding the development of reproductive cells in Pacific oysters and potentially other related species.
2. Discovery of specific gene markers for PGCs: In addition to vasa, the researchers found other genes, including a conserved germline marker called nanos, that were specifically expressed in putative PGCs. These markers could help scientists further study the processes governing reproductive cell development and differentiation in oysters.
3. Comprehensive analysis of early-stage oyster embryos: The research provided a detailed view of gene expression patterns in early-stage oyster embryos, identifying various cell types such as ciliary cells, muscle cells, and immune cells. This broad understanding of the complex processes involved in oyster development can contribute to our knowledge of marine biology, aquaculture, and conservation efforts.

3 Optimize delivery techniques of custom gene-regulating molecules to oyster embryos

3.1 Research Summary

A series of experiments were performed that aimed to optimize delivery of gene silencing molecules to Pacific oyster embryos. Initial experiments were performed with fluorescently labeled dextran (FLD) molecules as a cost-effective proxy for visualization of gene silencing molecules (i.e. morpholinos) in oyster embryos. Three modes of delivery were evaluated. First, FLD was co-introduced to unfertilized Pacific oyster oocytes in the presence of sperm. Compared to FLD alone, the FLD+sperm showed a high degree of florescence via fluorescence microscopy but was later determined to be localized only to the surface of the oocyte via confocal microscopy. The second mode of delivery that was explored was electroporation of fertilized oyster ooctyes in the presence of FLD. Two electroporation protocols, previously published were tested in two replicate trials. The results of these trials indicated that a proportion of 2 day old larvae were uptaking the FDL resulting in fluorescent larvae, however, survival was low and many developmental abnormalities were observed indicating there is room to optimize the electroporation protocols for Pacific oysters. The third mode of delivery was introduction of FLD in the presence of a peptide delivery vehicle called Endo-Porter (GeneTools). These trials identified an optimum Endo-Porter concentration for bivalves, but additional trials will be needed to optimize the delivery timing and concentration. Additional insights achieved during these trials included: 1) optimized protocols for plating oocytes/larvae to assess fluorescence, 2) optimized fixation protocols to view embryos via fluorescent and confocal microscopy, 3) optimization of the number early embryos (i.e. blastula stage) required to be able to reliably isolate mRNA for expression analyses.

3.2 Specific Research Activity

3.2.1 Dextran Testing

The purpose of this effort was to explore passive uptake of fluorescent labeled dextran in C.gigas oocytes in preparation for electroporation trials. The study involved reconstituting and aliquoting the dextran, exploring the functionality of the fluorescent scope, evaluating whether or not there is passive uptake of the dextran, incubating embryos in 12 well plates to evaluate their survival, and counting embryos.

The experiments involved hydrating oocytes and preparing labeled dextran, conducting dye trials to observe the uptake of the dye, and embryo plating. In the dye trials, the dye was mixed with only the oocytes initially and then with sperm and oocytes. The results showed that dye mixed with sperm and oocytes showed lots of fluorescence, whereas in cases where only oocytes were used for the trials, no fluorescence was observed. The study then involved embryo plating, where fertilized oocytes were counted and placed in a 12 well plate. The plates

were then observed for 48 and 96 hours to evaluate their viability. The embryos survived for the most part, and the results of this experiment can help inform future electroporation trials.

3.2.2 Endoporter Testing

We conducted a series of experiments to test the use of morpholinos in conjunction with fluorescent endo-porter for C. gigas oocytes. The impact of endo porter and morpholino on fertilized egg survival was explored through four different trials. Results indicated that 2uM endo porter concentration yielded a 10% survival rate in the control group. Trials 1 and 3 displayed higher survivorship rates (over 10% and around 50%, respectively) with no fluorescence, while Trial 2 resulted in all embryos dying with increasing fluorescence. Trial 4 had a low survivorship of 5% and no fluorescence, but the morpholino concentration was determined to be too high. The conclusion suggests testing lower morpholino concentrations alongside optimized endo porter concentrations for better outcomes.

3.2.3 Electroporation Testing

This scientific protocol aimed to study the potential of electroporation to deliver molecules into embryos. The experiment is divided into three phases: 1) Determined if embryos can be plated and kept alive, and if they autofluoresce under various wavelengths. 2) Explored electroporation parameters and the uptake of fluorescein-labeled dextrans. Parameters include dextran concentration, pulse shape, pulse time, number of pulses, buffer conductivity, pulse voltage, and egg density. Indicators were percentage of fluorescent embryos and viability assay. 3) The experimental design is based on treating fertilized eggs with different electroporation protocols and evaluating the outcomes in terms of fluorescence, viability, and development. Imaging challenges were addressed with protocol below (appendix) The approach has made progress in understanding the potential of electroporation for delivering molecules into bivalve embryos.

3.3 Appendix

3.3.1 Definitions

Dextran is a complex, branched polysaccharide derived from bacterial fermentation. It is composed of glucose units linked together and has various applications in biology, including as a molecular carrier for delivering substances into cells. In the context of molecule delivery into eggs, dextrans conjugated to molecules of interest (such as fluorescent dyes, proteins, or nucleic acids) can be microinjected into eggs or embryos, allowing researchers to study the distribution, dynamics, and function of these molecules in the developing organism.

Endo-Porter is a proprietary peptide-based delivery system designed to facilitate the uptake of molecules by cells. It works by allowing molecules that are typically unable to cross the cell membrane, such as nucleic acids and proteins, to be internalized by cells via endocytosis. In the case of eggs, endo-porter can be used to deliver molecules like morpholinos, siRNA, or other gene-modulating agents to study gene function and regulation during early development. This method is less invasive than microinjection and can provide more uniform delivery of molecules across the cells.

Morpholinos are synthetic, non-toxic, nucleic acid analogs that can specifically bind to target mRNA sequences through complementary base pairing. They are used to modulate gene expression by blocking translation (protein synthesis) or splicing of the target mRNA. Morpholinos are commonly used in developmental biology research to study gene function in eggs and embryos by knocking down or modifying the expression of specific genes. These molecules can be introduced into eggs or embryos through methods like microinjection or electroporation, or by using a delivery agent like endo-porter.

3.3.2 Protocol - Fixation of Embryos

This protocol describes a method for prserving speciemens for latter microscop and includes the preparation of a 4% paraformaldehyde (PFA) solution for the fixation of embryos, followed by a series of washing and dehydration steps, and storage in methanol at -20°C. The method is designed for use to allow us to characterize fluorescence (*ie* experimental results) up to 3 weeks after treatment.

Materials and Methods:

1. Preparation of 4% PFA Solution:

   a. Add 10 mL of 16% PFA (whole contents of a glass vial) to a 50 mL conical falcon tube.

   b. Fill the tube to 40 mL with RNase-free phosphate-buffered saline (PBS).

   c. Note the date the solution was made.

   d. Store at 4°C.

2. Fixation of Embryos:

   a. Concentrate embryos at the bottom of the tube, removing as much seawater as possible.

   b. Add ice-cold 4% PFA in PBS to the tube and incubate at 4°C for 2 hours with the tube on its side. Gently mix by inversion several times.

   c. Concentrate embryos at the bottom of the tube.

d. Remove fixative (discard in "PFA waste") and wash embryos five times in PBS + 0.1% Tween-20 (PTw). For each wash, collect embryos at the bottom of the tube, remove PTw, and add 1 mL fresh PTw.

e. Remove PTw with one wash in deionized (DI) water and proceed to dehydration.

3. Dehydration:

a. Remove DI water and add 30% methanol/70% PTw. Incubate at room temperature with the tube on its side for 5 minutes.

b. Remove solution and add 50% methanol/50% PTw. Incubate at room temperature with the tube on its side for 5 minutes.

c. Remove solution and add 70% methanol/30% PTw. Incubate at room temperature with the tube on its side for 5 minutes.

d. Remove solution and add 100% methanol. Incubate at room temperature with the tube on its side for 5 minutes.

e. Remove methanol and add new 100% MeOH.

4. Storage:

a. Store embryos in 100% MeOH at -20°C.

Notes:

- Addition of Tween-20 will prevent embryos from sticking together.
- Laying the tube of embryos on its side during the fixation step will allow more access of fixative to embryo cells.
- Embryos and larvae must be stored in methanol at -20°C at least overnight prior to ISH.