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# Examining lateral line development through CXCL14 modulation of CXCL12-CXCR4 mediated gene expression in *Danio rerio*

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**Examining lateral line development through CXCL14  
modulation of CXCL12-CXCR4 mediated gene expression in**

*Danio rerio*

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Monday, April 29, 2019

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## ABSTRACT

The lateral line is a mechanosensory system used by fish to sense the movement of water. It is evolutionarily related to the inner-ear in humans. For both organisms, the binding of the CXCL12 (SDF-1 ligand) to the CXCR4 receptor induces conformational changes needed to activate signal transduction. This signaling results in numerous cellular responses such as cell fate, chemotaxis, and gene transcription. Interestingly, researchers have found that another signaling molecule, CXCL14, can also bind to the CXCR4 receptor with high affinity (Tanegashima et al., 2013). As a result, we hypothesize that CXCL14 modulates CXCL12-mediated chemotaxis, presumably acting as an allosteric regulator.

We are concerned with the allosteric relationship between CXCL14 and CXCL12 and how those relationships affect gene expression in lateral line development. In order to study this interaction, zebrafish were used as our model organism. To examine the effects of CXCL14 on CXCL12-mediated gene transcription, zebrafish embryos were microinjected with CXCL14 antisense morpholino and incubated for a period of three timepoints: 24 dpf, 36 dpf, and 48 dpf, when lateral line development occurs. Our preliminary results suggest that the absence of CXCL14 affects the gene expression of *CXCL14*, *CXCL12*, *CXCR4*, *CXCR7*, *epcam*, *claudin*, and *snail1b* during lateral line development, thereby suggesting the allosteric capabilities of CXCL14. Further studies will be conducted to determine the degree to which CXCL14 affects the transcription levels of these genes.



# INTRODUCTION

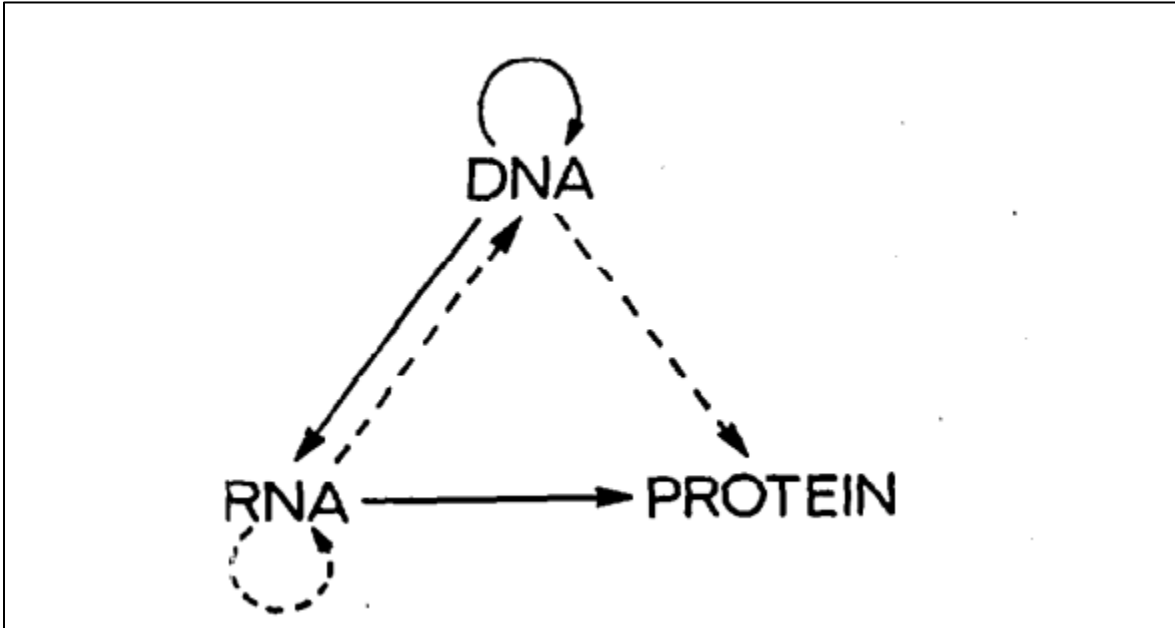
Zebrafish, or *Danio rerio*, are freshwater fish native to the Himalayan region. In order to properly detect and avoid predators, zebrafish rely on a lateral line system to sense movement in the water. The lateral line system is a mechanosensory system used by fish to detect water current stimuli. Similar to the inner-ear in humans, the lateral line system allows zebrafish the ability to “hear” their surroundings. This provides zebrafish with coordination and balance. For zebrafish, lateral line development is dependent on activation of the CXCL12 (SDF-1 ligand)-CXCR4 signaling pathway. Once activated, this signaling pathway results in numerous cellular responses such as chemotaxis, changes in metabolism, and/or changes in gene expression. In recent years, however, CXCL14 has been shown to potentially modulate the CXCL12-CXCR4 pathway (Tanegashima et al., 2013). In our lab, we are concerned with the extent to which CXCL14-mediated gene expression affects lateral line development.

## **Part 1- Genetic information and signaling pathways**

### *1.1. The central dogma and gene expression*

To begin, every form of life is bound together by a shared characteristic: genetic information. On a molecular level, the flow of genetic information from one macromolecule to another is known as the “Central Dogma of Molecular Biology” (Figure 1) (Crick, 1970). This process begins with the interactions between deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). These nucleic acids store and transfer the information needed to provide the cell with instructions for making proteins (Lodish et al., 2000). Proteins are important macromolecules

that are essential for all life. For example, proteins provide structural support in the form of muscles as well as immunological support with the production of antibodies.



**Figure 1. The central dogma model for molecular biology.** Solid arrows denote general transfers of genetic material whereas dotted arrows show special transfer of genetic material. Adapted from Francis Crick (1970).

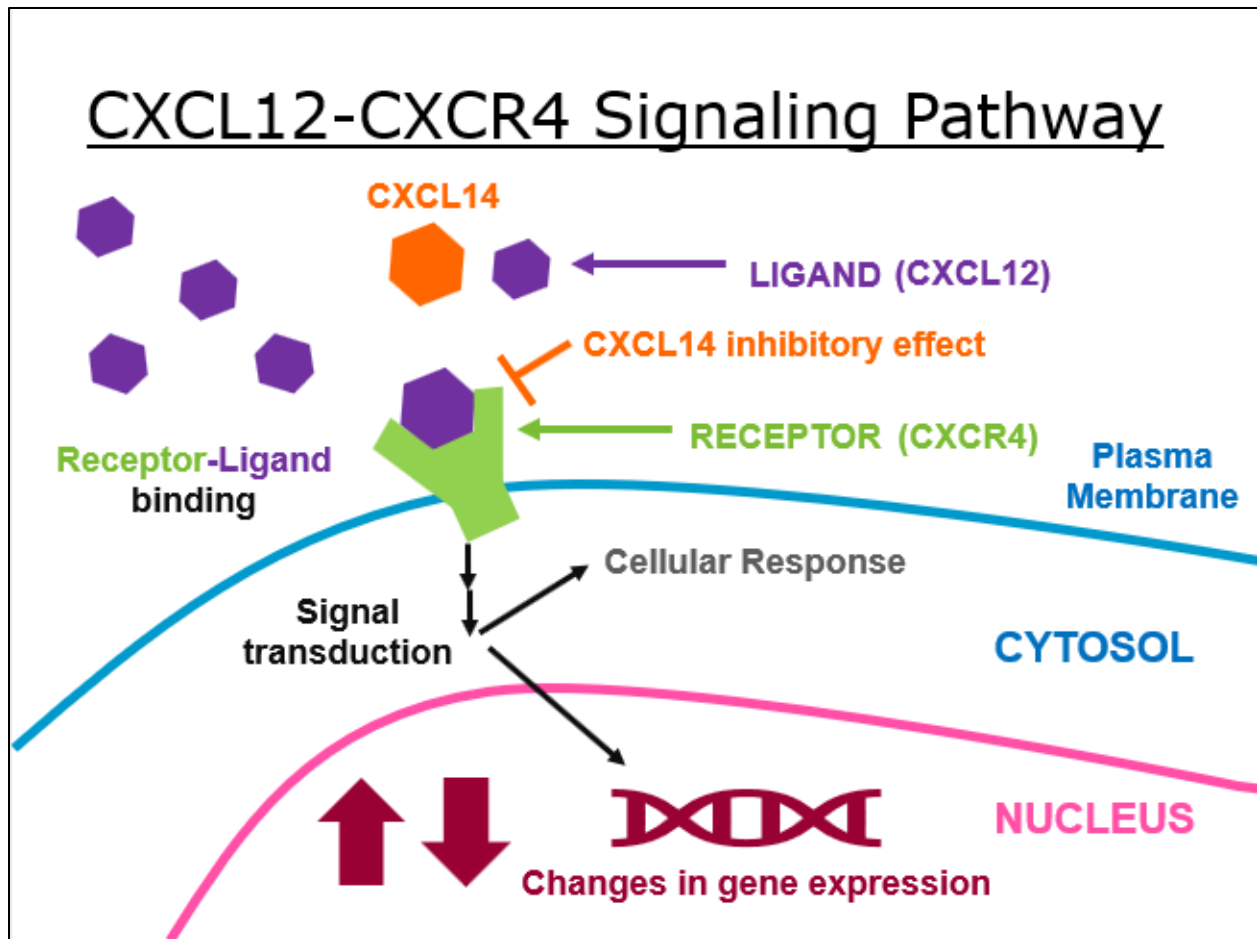
The genetic material stored in DNA is composed of four chemical bases: adenine (A), guanine (G), cytosine (C), and thymine (T) (Ptashne, 2004). Similar to letters forming words and sentences, these nucleotides serve as building blocks for the formation of nucleic acid polymers such as DNA or RNA (Ptashne, 2004). Likewise, the order in which these nucleotides are arranged determines the genetic information they convey. This process resembles how an arrangement of words changes the meanings they convey.

Embedded within DNA are genes which contain the nucleotide sequences needed to code for molecules. Some genes code for proteins with specialized functions such as transcription factors. When transcription factors bind to DNA, they can either activate or repress gene

transcription (Brooker et al., 2014). When the cell receives signals to produce proteins, the corresponding genes that code for them are activated. RNA polymerase transcribes genes into multiple copies of messenger RNA (mRNA) and, from there, ribosomes can now translate these mRNAs into the proteins needed for the cell (Ptashne, 2004). Once these proteins are synthesized, they can now carry out specialized jobs such as receiving and transmitting signals involved in regulating gene expression.

### *1.2. Signal transduction pathways and gene expression*

The cellular mechanisms that govern gene transcription are composed of several parts that come together to form signal transduction pathways. The start of signaling pathways begins with the interactions between a signaling molecule, known as a ligand, and its corresponding receiving molecule or, receptor (Brooker et al., 2014). Ligands are extracellular signaling molecules with a high affinity for their receptors; meaning they can recognize and bind to one or at most a few target receptors (Alberts et al., 2002). These target receptors are found on the surface of cells. When ligands bind to their respective receptors, communication occurs where extracellular signals are transduced to the molecules that govern intracellular responses (Figure 2).



**Figure 2. General schematic of the CXCL12-CXCR4 cell signaling pathway.** When chemokine ligand 12 (CXCL12) binds to its corresponding chemokine receptor (CXCR4), a signal transduction pathway is activated. This leads to a series of cellular responses such as changes in gene expression. Gene transcription can either be upregulated or downregulated, causing an increase or decrease in gene expression respectively. When chemokine ligand 14 (CXCL14) binds to the same receptor, CXCL12-mediated signaling is inhibited.

In order to produce a cellular response, there must be an interaction between ligands and receptors. Namely, when ligands bind to their receptor, a conformational change must occur wherein the shape or activity of the receptor is changed (Brooker et al., 2014). These conformational changes activate the ligand-receptor complex and allow extracellular cues from the ligand to be translated into signals that can be fed into the appropriate signal transduction pathway (Brooker et al., 2014). Once these signals are transmitted inside the cell, molecules

within the signaling pathway are activated. Thereafter, the signal grows stronger until a cellular response is produced.

Signaling pathways are composed of several molecules responsible for activating or repressing a series of cellular responses (Brooker et al., 2014). As mentioned, the cell must receive word of external stimuli through ligand-receptor binding. In response to extracellular stimuli, factors that regulate cellular responses are activated (Gomperts et al., 2002). More specifically, when the cell receives external cues, signals propagate to proteins residing in the appropriate signal transduction pathway(s). These proteins undergo a series of changes in order to send and amplify the signal. This is referred to as a signaling cascade (Brooker et al., 2014). From one factor to the next, the signal cascades down a signaling pathway where it becomes amplified. As more and more proteins are activated, the signal becomes stronger. Once these signaling cascades take root, the signal continues to travel down a pathway until the appropriate cellular response is produced. In my lab, we are focused on one very important cellular response- gene transcription.

Signaling pathways give rise to a wide variety of responses that regulate important cellular functions such as gene transcription or cell differentiation, wherein the cell can differentiate to fit the needs of the organism (Berg et al., 2002). When signaling pathways occur, proteins that regulate gene transcription, also known as transcription factors, are activated (Brooker et al., 2014). These transcription factors ultimately determine whether certain genes are turned on or off. Biochemically speaking, gene expression is regulated by signal transduction pathways and the proteins that either activate or repress them.

It is important to note that these pathways can affect gene expression in one of two ways: during development and/or cell differentiation. As an organism develops, certain genes are

turned on or off. This allow cells to differentiate into what the organism needs (Brooker et al., 2014). From there, an embryo can develop properly without having too much or too little of a certain product. Although regulation is a key function of cells, there will always be exceptions; namely, cancer. In terms of malignant growth, cancerous cells multiply under conditions their normal counterparts do not. This process is caused in part by certain genes being turned on or off when they should not be (Ptashne, 2004).

### *1.3. Cancer biology*

Since the first documented case around 1600 B.C.E., cancer has become a popular area of study for human health (Sudhakar, 2009). In recent years, scientific research has expanded the area of oncology, the study of cancer, to include more research for drug therapies that can target and combat cancer. More often than not, cancerous cells take advantage of damaged DNA in normal cells to utilize cell machinery for their own device (Sudhakar, 2009). While some cancer cells continue to grow and develop without regulation, others take advantage of the circulatory system and travel through the bloodstream (Brooker et al., 2014). When this occurs, cancer cells circulating in the blood can come into contact with, and spread to, other parts of the body. This is referred to as metastasis (Brooker et al., 2014). Through the circulatory system, cancer can spread and grow in areas apart from the site of origin. One way the body defends against these cells is through immunosurveillance.

#### *1.4. CXCL12 and CXCL14 are closely related CXC-family chemokines*

Immunosurveillance is predominantly regulated by a family of chemokines (CXC). Chemokines are small proteins that heavily regulate cell migration for both organogenesis (the formation of organs) and immunosurveillance (Bacon et al., 2000). In particular, these CXC-chemokines are chemotactic, meaning they cause cells to respond to chemical stimuli in their environment. In doing so, chemokines promote cell migration for processes such as organogenesis and immunosurveillance to occur (Collins et al., 2017).

Most notable among this family are chemokine-ligand 12 (CXCL12) and chemokine-ligand 14 (CXCL14), both of which are considered primordial chemokines due to their evolutionarily conserved nature. Found among many different organisms, such as humans and zebrafish, these ligands play an important role in embryogenesis (the formation and development of an embryo) and immunosurveillance (Hara et al., 2012; Collins et al., 2017). As a result, zebrafish are an important model organism for analyzing signal transduction pathways and assessing their role in different cellular responses; many of which, are found in humans.

#### *1.5. CXCL12-mediated signaling pathways*

As mentioned previously, in order to promote cellular responses for processes such as embryogenesis, a signaling cascade must occur. There are two known receptors for CXCL12: chemokine-receptor 4 (CXCR4) and chemokine-receptor 7 (CXCR7) (Tanegashima et al., 2013a). When CXCL12 binds to either CXCR7, or more importantly to CXCR4, a signaling cascade is activated within the cell. As a result, a cellular response is produced that triggers

important processes such as the homing of lymphocytes or the migration of primordial germ cells and neural progenitors (Tanegashima et al., 2013a; Eckert et al., 2018).

Lymphocytes, also known as B cells or T cells, are a type of white blood cell responsible for specific immunity (Brooker et al., 2014). Lymphocytes send signals to cells in response to damage or infection, thereby playing a crucial part in regulating our immune system (Brooker et al., 2014). Migration of cells is also observed during embryonic development. During embryogenesis, cells migrate to where they are needed (Brooker et al., 2014). This allows for the organism to develop properly for maturation. In addition, neural progenitors can migrate and develop into neuronal cells and glial cells. These cells help maintain homeostasis and provide support and protection for neurons (Eckert et al., 2018).

Unfortunately, if cells experience any form of damage, they are vulnerable to opportunistic pathogens, programmed cell death (apoptosis), or cancer. During DNA repair/DNA-damage pathways, cancer progression can occur. This is due to high levels of genomic instability and an increase in mutation rate (Turgeon et al., 2018). In addition to DNA-repair pathways, it is known that other signaling pathways play a role in cancer proliferation. In particular, the CXCL12–CXCR4 signaling pathway has been shown to promote cancer growth and metastasis (Tanegashima et al., 2013a; Tanegashima et al., 2013b; Eckert et al., 2018).

In recent years, researchers have discovered that the CXCL12–CXCR4 signaling pathway is manipulated by several cancer types to promote cell growth and metastasis (Tanegashima et al., 2013a). As a result, elevated levels of CXCR4 and CXCL12 have led to a poor prognosis for cancer patients (Tanegashima et al., 2013b; Eckert et al., 2018). With elevated levels, there is increased CXCL12-CXCR4 signaling, which has been shown to contribute to



malignant tumor growth, metastasis, neoplastic transformation, and infiltration (Eckert et al., 2018).

The CXCL12–CXCR4 signaling pathway has been attributed to the close relationship between malignant growth in over 20 different cancers, including: lung, breast, and ovarian cancers (Tanegashima et al., 2013b). Researchers have studied the CXCL12-CXCR4 pathway in hopes of unlocking ways to combat and repress cancer growth. Consequently, in the process of analyzing CXCL12-CXCR4 signaling, it appears another chemokine-ligand may hold the answer to suppressing tumor growth.

Generally, it is understood that CXCL12 binds to CXCR4 to promote cell migration and cell differentiation. Although these processes aid in organismal development, the CXCL12-CXCR4 pathway can be used by opportunistic cancers to develop without any form of regulation. In more recent years, however, it has been discovered that chemokine-ligand 14 (CXCL14) plays a very important regulatory role in the CXCL12-CXCR4 pathway.

### *1.6. CXCL14 as an allosteric modulator*

In the field of cell biology, it is commonly accepted that ligands are matched to their respective receptors. Allosteric regulators are molecules which influence (or modulate) the signaling capabilities of ligands. More specifically, allosteric regulators can have an impact on a ligand's ability to activate or repress a target protein's function (Rothman et al., 2015). In the case of CXCL14, this chemokine may act as an allosteric modulator.

Similar to its counterpart (CXCL12), CXCL14 is a primordial chemokine. While CXCL14 is an evolutionarily conserved chemokine, there does not appear to be a specific receptor it binds to. Although a unique receptor for CXCL14 has not been identified, the ligand

is known to regulate a variety of normal cellular responses (Collins et al., 2017). For example, CXCL14 has been shown to regulate metabolism, cell migration, and immunosurveillance.

In terms of cancer, CXCL14 has been shown to have both tumor-suppressing or tumor-supporting functions (Otte et al., 2014). CXCL14 expression varies among different cancer types. As a result, CXCL14's role in tumor suppression, and progression, differs (Collins et al., 2017). For some cancers, CXCL14 gene expression is downregulated (Hara et al., 2012). This contrasts with high levels of CXCL12 gene expression in many cancers. One possible explanation for decreased CXCL14 expression in some cancers is that loss in CXCL14 results in a loss of immunosurveillance of cancer cells. Therefore, a decrease in CXCL14 expression promotes cancer cell survival and proliferation.

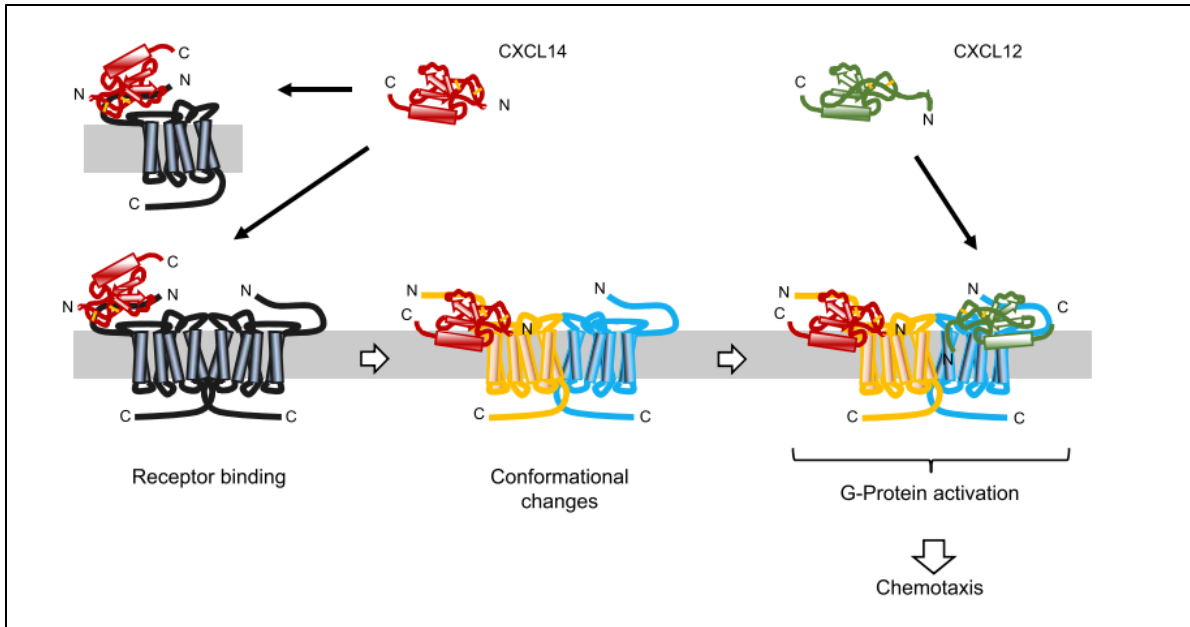
While a unique receptor for CXCL14 has not been identified, it has been shown that CXCL14 can bind to CXCR4, the CXCL12 receptor, with high affinity (Hara et al., 2012; Tanegashima et al., 2013a; Tanegashima et al., 2013b; Collins et al., 2017). In doing so, CXCL14 interferes with CXCL12 signaling through its receptor. More specifically, CXCL14 inhibits CXCL12's ability to initiate cell migration of bone marrow derived hematopoietic progenitors (Tanegashima et al., 2013a; Tanegashima et al., 2013b). Hematopoietic progenitor cells give rise to blood cells, B-cells, and T-cells. Whereas B-cells and T-cells aid in immunological responses, blood cells can be divided into two primary groups. When red blood cells are made, they exclusively transport oxygen to different parts of the body. On the contrary, white blood cells can differentiate into numerous types of immune cells to target pathogens.

When the bone marrow is damaged, abnormal leukocytes can enter the lymphatic system and use it to divide. In this situation, cancer cells can repurpose the CXCL12-CXCR4 signaling pathway to promote tumor growth and metastasis (Tanegashima et al., 2013a). By using the

CXCL12-CXCR4 pathway, cancer cells can continue to grow and divide without interruption. In addition, their survival is facilitated by CXCL12's ability to promote metastasis and cell migration.

Several studies have demonstrated CXCL14's ability to inhibit CXCL12-mediated processes by binding to CXCR4. As a result, researchers have proposed that CXCL14 is a natural inhibitor of CXCL12 by highlighting its ability to "fine-tune" the CXCL12-CXCR4 pathway (Tanegashima et al., 2013a). Due to the conservation of CXCL12 and CXCL14 sequences across organisms, it can be assumed that these two chemokine-ligands have evolved together to assume regulatory functions within similar pathways (Tanegashima et al., 2013a). As a result, we can assume that CXCL14 possesses the ability to allosterically modulate the CXCL12-CXCR4 signaling pathway.

CXCR4, being a receptor, is found on the surface of cells; a location which facilitates contact with external signals. CXCR4 contains transmembrane helices which allow extracellular cues to communicate with signaling molecules inside the cell. On a chemical basis, CXCL12 contains an N-terminal region with the ability to bind to the transmembrane helices of CXCR4 (Tanegashima et al., 2013b). When this chemical interaction occurs, the CXCL12-CXCR4 signaling pathway is activated. Similarly, a specific domain within CXCL14 can bind to CXCR4. In doing so, CXCL14 modulates CXCL12-CXCR4 signaling (Figures 2 and 3).



**Figure 3. Model explaining how CXCL14 can synergize with CXCL12 in the induction of CXCR4-mediated chemokine responses.** Conformational states can be influenced by ligand binding (shown by the shift from black to yellow conformation upon CXCL14 binding). CXCL14 binding induces allosteric changes in partner molecules that are present in CXCR4 dimers, thereby lowering the threshold of receptor activation by the functional ligand CXCL12 (shown here by the shift from black to blue conformations in the partner molecule). Adapted from Collins et al. 2017.

The plasma membrane is a physical barrier which envelopes the cell and separates the intracellular environment from its extracellular surroundings (Brooker et al., 2014). When the N-terminal region of CXCL12 comes into contact with CXCR4, a conformational change occurs where molecules on the intracellular side of the plasma membrane receive signals from extracellular stimuli. From there, signal transduction pathways are activated, and cellular responses occur. Without this interaction, conformational changes do not occur, and signal transduction is not activated. This process occurs in the presence of CXCL14.

CXCL14 has the ability to inhibit CXCL12-CXCR4 mediated activity in humans. When approaching this from a pharmacological perspective, CXCL14 can potentially be used as a form of drug therapy to combat CXCL12-mediated cancer growth. Due to its allosteric nature,

CXCL14 can be designed to out-compete CXCL12. In doing so, CXCL14 can be more apt to bind to CXCR4, and with higher affinity. By preventing elevated levels of CXCL12 from circulating and encountering CXCR4, CXCL14 can be designed to efficiently inhibit CXCL12-mediated chemotaxis, thereby reducing cancer growth.

Many issues arise when undertaking a project of this caliber. Namely, without an identifiable receptor, potential CXCL14-stimulated signaling pathways are not fully understood. In order to design an effective form of drug therapy, we need to be aware of any competing reactions that could potentially interfere with CXCR4-CXCL14 binding. As a means of obtaining a better grasp of CXCL14 function, we must turn to model organisms for help.

## **Part II- Zebrafish and the lateral line system**

### *1.1. Zebrafish as a model organism*

Model organisms are widely studied species that are used to enhance our understanding of human health. These model organisms are used for research due to their reproductive nature, ability to maintain in a laboratory setting, and their value in experimental design (NIGMS, 2018). From mice, to worms, to fruit flies, the use of model organisms has led to extensive research in the field of biology. It wasn't until George Streisinger, however, that zebrafish were brought into the spotlight (Parichy, 2015). Later dubbed the "founder of modern zebrafish research," Streisinger published a series of papers where he used zebrafish as a model organism to investigate biology relevant to human health.

Zebrafish, or *Danio rerio*, are small freshwater fish native to the Himalayan region. Since their debut in the 1980's, zebrafish have emerged from the water as a novel model organism

(Parichy, 2015). Zebrafish are ideal model organisms for several reasons. They are small and easy to maintain. They have external fertilization and can produce hundreds of embryos per mating. Zebrafish embryos undergo rapid, external development and are transparent through early development (Adams et al., 2018). This allows researchers a convenient way to visually analyze the process of embryogenesis (Hsieh et al., 2002). As a result, zebrafish are used in research to study development, disease, and physiology.

Following their increase in popularity, extensive work has been done on zebrafish, including sequencing of their genome. Through genome sequencing, it has been revealed that zebrafish contain roughly 70% of the same genes as humans (Wellcome Genome, 2014). Moreover, many of these genes are associated with disease in humans. Researchers can use molecular tools, such as morpholinos, to silence specific genes and analyze their function in zebrafish in order to assess their role in humans (Adams et al., 2018). Morpholinos are a type of stable antisense molecules that will complement mRNA molecules and prevent translation of a functional protein product. This project utilized a morpholino targeted to CXCL14 and focused on the effects of loss of CXCL14 function on the CXCL12-CXCR4 signaling pathway. More specifically, its role in gene expression and embryonic development of the lateral line system.

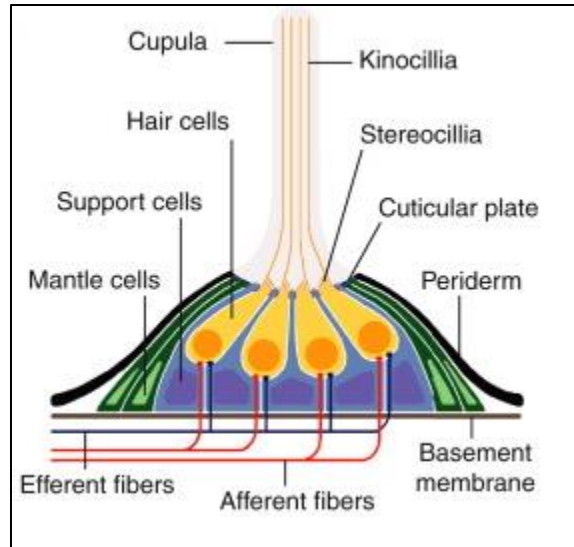
## *1.2. The lateral line system*

The lateral line system is a mechanosensory structure found in fish and is evolutionarily related to the inner ear in humans. The lateral line system is a sensory network used by fish to detect movements of water (Coombs et al., 2016). However, in order to sense water current stimuli, zebrafish depend on neuromasts present in the lateral line (Chitnis et al., 2012). Neuromasts consist of mechano-sensory hair cells that are innervated by sensory neurons

branching to and from the brain (Ghysen et al., 2004). For zebrafish to respond to their environment, the brain needs to receive the stimulus associated with movement. Hair cells help facilitate this communication.

Hair cells are so-called because they extend hair like ciliary bundles embedded within a gelatinous cupula that is in contact with the water (Figure 4). Therefore, each neuromast has a cupula which bends with movement of the water (Chitnis et al., 2012). Due to its gelatinous nature, any movement in the water causes the cupula to bend in the direction of the stimuli (Ghysen et al., 2007). As a result, the ciliary bundles also move. This collection of movement, in both the cupula and ciliary bundle, causes a depolarization of the hair cells.

Since hair cells are innervated by sensory neurons (afferent neurons), this depolarization is passed on to the sensory neurons which transmit information regarding the movement to the central nervous system (CNS) (Chitnis et al., 2012). When the brain processes this stimulus, it sends a signal back to the hair cells as well as to muscles to move in response to the stimuli via efferent neurons. Zebrafish use their lateral line system to translate water current stimuli into signals that lead to behaviors such as obstacle and/or predator avoidance, schooling, or orientation to a water current (Montgomery et al., 1997).

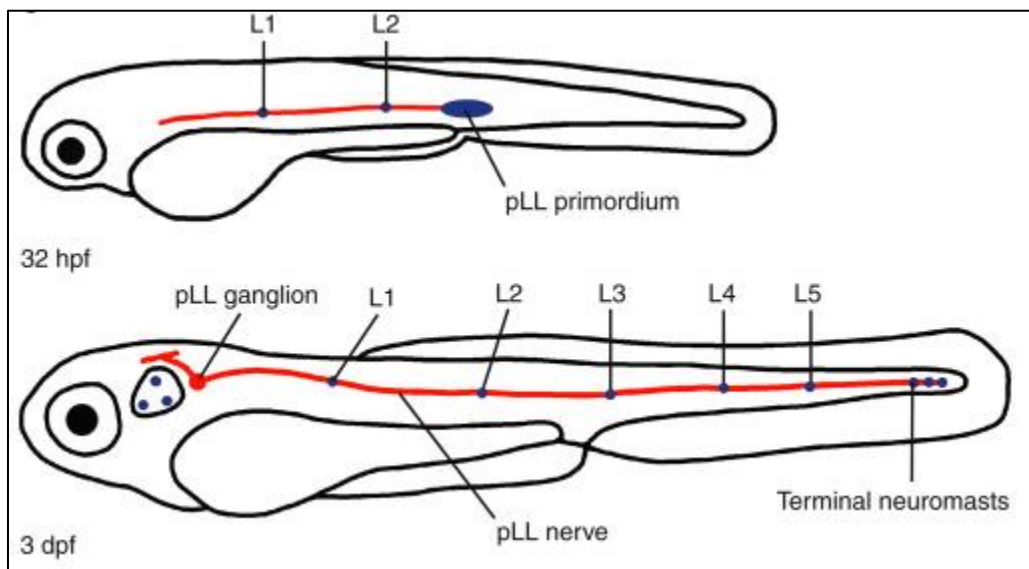


**Figure 4. Structure and innervation of a hair cell.** Hair cells are embedded within a gelatinous cupula that is in contact with water. In the event of water movement, cupulas bend in the direction of the stimuli. The movement in both cupulas and ciliary bundles causes a depolarization. This depolarization is passed onto sensory neurons via afferent fibers. When the brain processes this stimulus, a signal is sent back to the hair cells via efferent fibers. Adapted from Current Biology.

The lateral line system can be broken down into two parts: the anterior lateral line (ALL) and the posterior lateral line (PLL). Neuromasts on the head of the fish form the ALL whereas neuromasts extending from the head towards the tip of the tail, form the PLL (Ghysen et al., 2012). The posterior lateral line develops from a placode near the otic (ear) vesicle. Placodes are formed during embryonic development from thickenings of the surface cells (ectoderm) and eventually give rise to cranial ganglia and olfactory epithelium. In the case of the lateral line placode, cells from the placode will detach from and begin migrating to form the posterior lateral line primordium (PLLP). In doing so, the cells will migrate along a path following the horizontal myoseptum, a layer of connective tissue separating the dorsal (back) and ventral (belly) musculature and travel to the tip of the tail.



As the PLL primordium migrates, neuromasts are deposited along the way (Figure 5) (Ghysen et al., 2004). The cells that remain in the PLL placode are used to form the neurons of the lateral line ganglion, which will serve as the communication center between the brain and neuromasts. Sensory neurons in the lateral line ganglion extend growth cones that follow the migrating primordium to innervate the sensory hair cells of deposited neuromasts. This process gives rise to the lateral line sensory system and its mechanosensory structure (Metcalf et al., 1985; Chitnis et al., 2012).



**Figure 5. Posterior lateral line (PLL) development.** Neuromasts extending from the head towards the tip of the tail form the PLL. The PLL develops from a placode near the otic (ear) vesicle. Cells from the placode will detach from and begin migrating to form the posterior lateral line primordium (PLL). As the PLL primordium migrates, neuromasts are deposited along the way. The cells that remain in the PLL placode are used to form the neurons of the lateral line ganglion. Adapted from Current Biology.

### *1.3. CXCL12-CXCR4 and lateral line development*

In zebrafish, the CXCL12-CXCR4 signaling pathway plays an important role in lateral line development. For the posterior lateral line primordium to migrate from just posterior of the otic (ear) vesicle to the tip of the tail, chemokine signaling must occur. CXCR4, located on the surface of migrating cells, binds to CXCL12, which is found along the horizontal myoseptum. The presence of CXCL12 is attributed with providing a prospective pathway for the primordium to migrate on and to establish where the posterior lateral line will develop (Ghysen et al., 2004). This is due to CXCL12's chemotactic nature. In studies, it has been found that loss of CXCL12 expression results in failure of the PLL primordium to migrate along the horizontal myoseptum (Ghysen et al., 2004; Valentin et al., 2007).

Given the importance of the lateral line system as a mechanosensory structure and CXCL12's role in its formation, it is important to understand how signaling pathways such as the CXCL12-CXCR4 is regulating PLL development. In recent years, researchers have discovered that CXCL14 interacts with CXCR4 to modulate CXCL12-mediated chemotaxis in other biological contexts. This suggests that CXCL14 can function this way during development to regulate the CXCL12-CXCR4 signaling pathway. In our lab, we are focused on whether CXCL14 regulates lateral line development by attenuating the migratory signal mediated through CXCL12-CXCR4 signaling pathways via allosteric modulation.

## MATERIALS AND METHODS

### 2.1. Zebrafish husbandry

Zebrafish (*Danio rerio*) of the AB wildtype-strain were obtained from ZIRC- Zebrafish International Resource Center (Eugene, OR, USA) and housed in six separate 2.8-liter tanks (Aquaneering, San Diego, CA, USA). Each tank housed three males and three females. Tanks were separated according to birthdates. Zebrafish were maintained at 28.5°C with a 10-hours dark, 14-hours light cycle. Zebrafish were fed TetraMin tropical flakes (Blacksburg, VA, USA) ad libitum and had continuous access to filtered, aged tap water using the Pentair Shurflo water pump (Minneapolis, MN, USA) and Aquaneering water filter system (San Diego, CA, USA). Embryos were incubated in 1x E3 buffer at 28.5°C. Developmental stages were determined by embryo morphology and hours post-fertilization (hpf). See Appendices 1 and 2 for detailed protocols.

### 2.2. Microinjection

Zebrafish embryos were injected at the one-cell stage with 1 nl of 0.3 uM CXCL14 antisense morpholino (MO) in phenol red buffer (5'- CCGTACTACAGCGATTCATCCCCAA-3'). CXCL14-MO was obtained from Gene Tools (Gene Tools LLC., Philomath, OR, USA) (Appendix 2). For controls, zebrafish embryos were microinjected with 1 nl phenol red buffer and, as an additional control, not microinjected. Zebrafish embryos were maintained at 28.5°C for a series of three incubation periods: 24 hours post-fertilization, 36 hours post-fertilization,

and 48 hours post-fertilization, at which time viable embryos were assessed and total RNAs were extracted. See Appendices 2 and 3 for detailed protocols.

### *2.3. Primer Design*

Two different sets of gene specific primer pairs were designed using NCBI Primer-BLAST (National Center for Biotechnology Information, Rockville Pike, MD, USA). To examine whether CXCL14 knock-down affects gene expression, we designed primer pairs based off possible genes affected by, or downstream of, either CXCL14 or CXCL12. This set of gene specific primer pairs were used for qPCR as follows:  $\beta$ -actin and GAPDH were used as reference control genes whereas NF-kB, SCX-a, pmp22-b, mpz, mbp, OSR1, OSR2, and COL17-A1b were target genes of interest (Table 1).

Based off inconclusive data from primer set #1 (Appendix 5), the research question was reformulated to examine the relationship between CXCL14 knock-down and embryonic development. To determine whether CXCL14 knock-down affects gene expression, we designed primers based off genes expressed during lateral line development. This set of gene specific primer pairs were used for qPCR as follows:  $\beta$ -actin was used as the reference gene whereas CXCL14-a, CXCL12-a, CXCR4-b, CXCR7-b, snail-1b, cldn-2, and epcam were target genes of interest (Table 2). See Appendices 4 and 5 for detailed protocols.

<b>Primer Design Set #1: Sequence Information</b>				
	<b>Gene</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>	<b>Fragment size</b>
<b>Control</b>	B-actin	5-TCACCACCACAGCCGAAAG-3	5-AGAGGCAGCGGTTCCCAT-3	98bp
<b>Control</b>	GAPDH	5-GTGTAGGCGTGGACTGTGGT-3	5-TGGGAGTCAACCAGGACAAATA-3	121bp
<b>DT of CXCL14</b>	NF-kB	5-GCTCCAGGATGACGTTCAAGTA-3	5-CCAGAAAGTCCCCTTGAGGT-3	201 bp
	SCX-a	5-TCAGGGAGGGATGAGAGCAG-3	5-TCTGCTCCAGAGAACCGAGA-3	335 bp
	Pmp22-b	5-TGGAAACGAGGAGCAGAACC-3	5-AGTGAACCTGAGAGGAGGGT-3	496 bp
	Mpz	5-ATTGCGTCCTTAGCCCCATC-3	5-GTGTTTGTATCCTCCAGCCTCT-3	177 bp
	Mbp	5-TGTCCGAGTCAAGTTGCTACA-3	5-GCTTTTGGTTGGGCAGTCAG-3	316 bp
<b>DT of CXCL12</b>	OSR1	5-CCTCAATCCCCTGTTCCCC-3	5-AGGCAGGTATGAGCAGGAATG-3	484 bp
	OSR2	5-AGTCTTACTGCCATTCCCG-3	5-AACCGCTCAAACCTGTGTGTTTC-3	116 bp
	COL17A1b	5-TCACTTTGTCACTAATGCCGAT-3	5-TCACCCTTTTCTCCCCTTGG-3	327 bp

**Table 1. Sequence information for primer pair set #1.** Primer pair sequences were designed for genes potentially affected by, or downstream of, either CXCL12 or CXCL14. These primer pairs were used for qPCR experiments. All primer pairs were designed using NCBI Primer BLAST. For more information, see Appendix 4.

<b>Primer Design Set #2: Sequence Information</b>				
	<b>Gene</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>	<b>Fragment size</b>
<b>Control</b>	B-actin	5'-TCACCACCACAGCCGAAAG-3'	5-AGAGGCAGCGTTCCCAT-3	98bp
<b>Control</b>	GAPDH	5'-GTGTAGGCGTGGACTGTGGT-3'	5'-TGGGAGTCAACCAGGACAAATA-3'	121 bp
<b>Ligands</b>	CXCL14-a	5'-TGCAGATGCACAAGAAAAGG-3'	5'-GGCTTCAAACGTCTGTGTT-3'	225 bp
	CXCL12-a	5'-TTCATGCACCGATTCCAAC-3'	5'-TGTTGATGGCGTTCTTCAGG-3'	222 bp
<b>Receptors</b>	CXCR4-b	5'-TACGATGTAAGTTGGCTTGTGA-3'	5'-CTGACTGAGAGGTCGCAAAG-3'	435 bp
	CXCR7-b	5'-GAGTTGCCACCACACAAAAGG-3'	5'-TGTGCTTAATTGGTCCCTGC-3'	396 bp
<b>Genes expressed in neuromasts and primordium</b>	Cldn-2	5'-GCGTTTATTGATTGCAGGCG-3'	5'-TTGCTGCCGTTTTATGTGC-3'	353 bp
	Snail-1b	5'-CGCTGAAGTTTCGAGGGGAT-3'	5'-CAGTGTTTGCAGTGGAAGGC-3'	485 bp
	Epcam	5'-CTGCCCTGTTTTGGAATGG-3'	5'-GCGTTTGGAAAACGAGACCTT-3'	375 bp

**Table 2. Sequence information for primer pair set #2.** Primer pair sequences were designed for genes expressed during lateral line development. These primer pairs were used for qPCR experiments. All primer pairs were designed using NCBI Primer BLAST. For more information, see Appendix 4.

#### *2.4. Real-time quantitative polymerase chain reaction (RT-qPCR)*

Total RNAs were extracted from 50 embryos at various embryonic stages: 24, 36, and 48 hpf using Trizol (Zymo Research, Irvine, CA, USA) reagent. RNA concentrations and purity were determined using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA strands (cDNAs) were synthesized from 1.5 ug of total RNA using the Easy-script reverse transcription system (Lamda Biotech Corporation, Ballwin, MO, USA). cDNAs were then amplified with gene specific primers and SYBR green reagents (Promega Corporation, Madison, WI, USA) in a 7500 real-time quantitative PCR system (Applied Biosystems, Foster City, CA, USA).

Quantitative polymerase chain reactions were used to quantify mRNA gene expression in each sample by measuring emitted fluorescence intensity. Reactions were performed under the following cycling conditions: pre-denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute, then a melting curve cycle at 95°C for 15 seconds, 60°C for 1 minute, 95°C for 30 seconds, and 60°C for 15 seconds to confirm amplification specificity. Reactions were conducted as single runs, duplicates, and triplicates (Appendix 4). qPCR results were used to calculate the variation of gene expression between two samples, also known as “relative gene expression.” Amplification products were then electrophoresed on 1% agarose gels. See Appendix 4 for detailed protocols and Tables 1 and 2 for primer pairs.

#### *2.5. Calculations and statistical analysis*

Relative gene expression levels for each sample were determined using the  $2^{-\Delta\Delta C_t}$  method with either GAPDH or  $\beta$ -actin as the reference gene.  $C_T$  values were averaged to calculate means and standard deviation of means per sample. To compare the effects of microinjection on gene

expression, data were presented as Log<sub>2</sub> mRNA fold-change levels of sham-injected embryos relative to non-injected embryos. To compare the effects of morpholino knock-down on gene expression, data were presented as Log<sub>2</sub> mRNA fold-change levels of morpholino-injected embryos relative to sham-injected embryos. All numerical data are presented as relative gene expression  $\pm$  standard deviation.

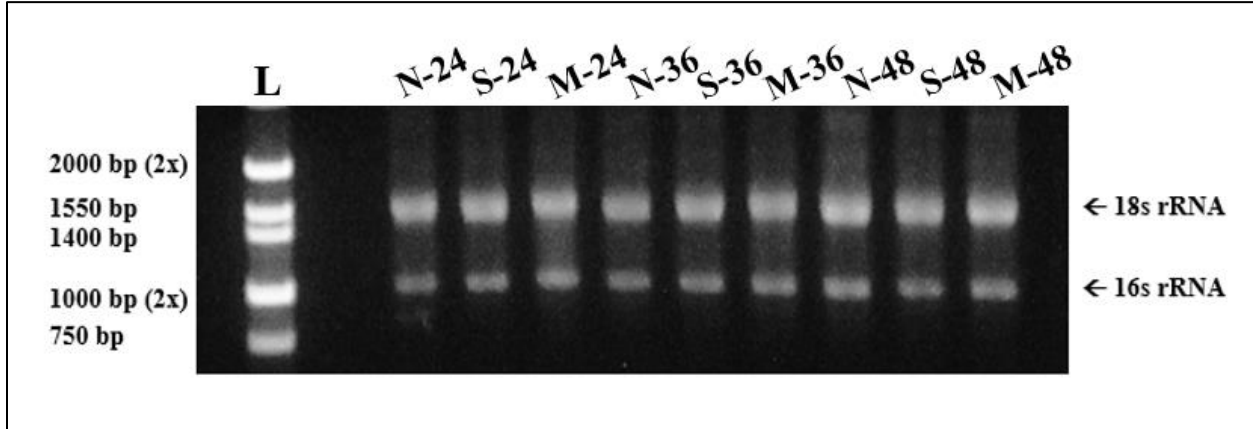
$\Delta C_T$  values were transformed and analyzed by a Mann-Whitney test to determine the statistically significant difference between non-injected and sham-injected embryos.  $\Delta C_T$  values were also transformed and analyzed by a Mann-Whitney test to determine the statistically significant difference between sham-injected embryos and morpholino-injected embryos. P-values were calculated assuming non-parametric distribution due to limited numbers of samples. All data were analyzed using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). See Appendix 4 for detailed protocols.



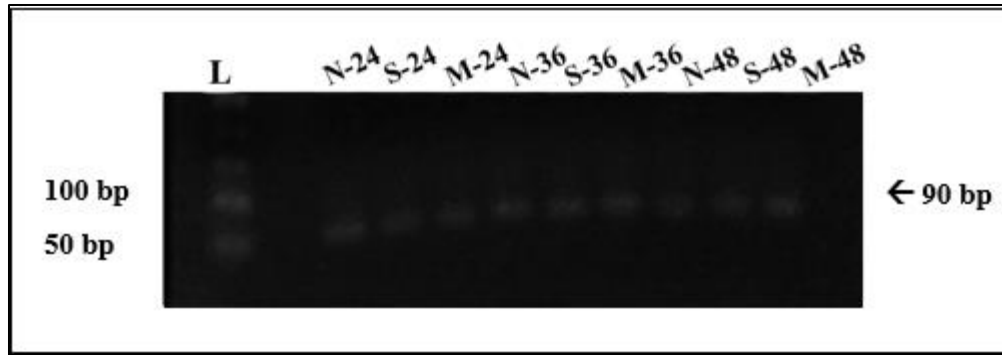
## RESULTS

### 3.1. Gel electrophoresis for amplified PCR products

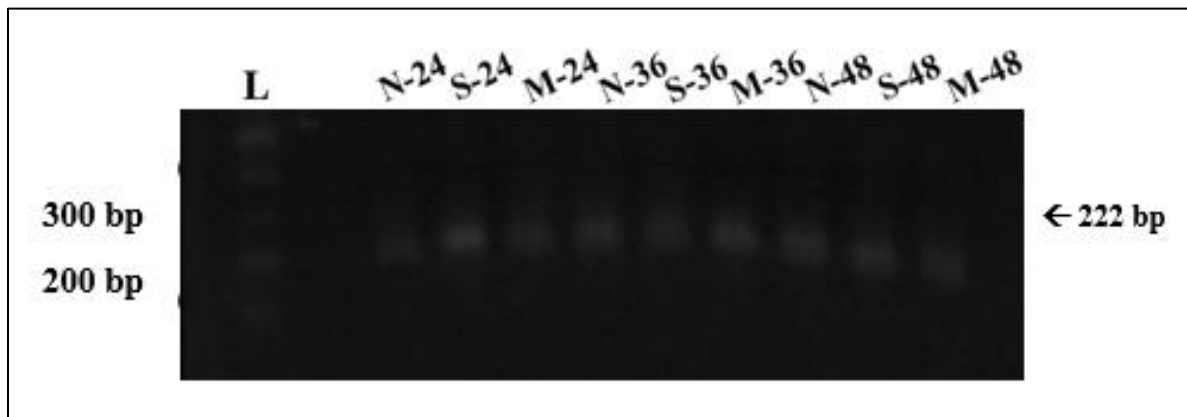
Gel electrophoresis was used to determine RNA and primer pair quality. For RNA gel electrophoresis, the 18S rRNA and 16S rRNA bands are clearly depicted at 1550 bp and 1000 bp, respectively. Clear and distinct bands indicate RNA purity, which coincides with NanoDrop results. There does not appear to be any RNA degradation or impurities, therefore the RNA extracted from each time point were used for cDNA synthesis reactions.



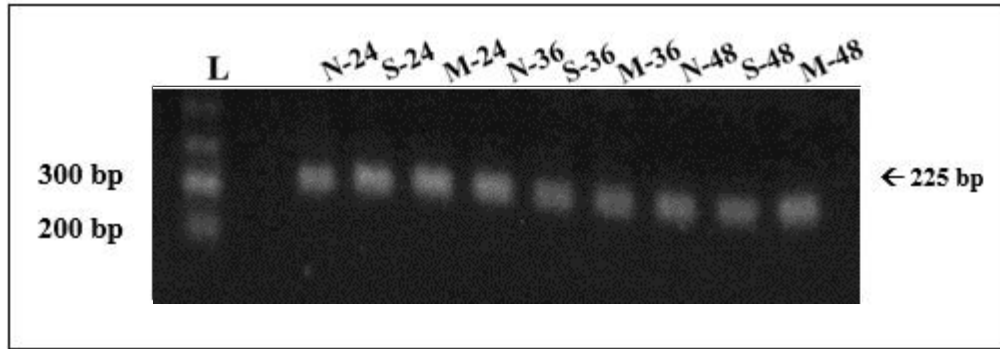
**Figure 6. Gel Electrophoresis for RNA samples from *Danio rerio* zebrafish at 24, 36, and 48 hpf.** The DNA ladder is displayed as “L.” RNA extracted from non-injected embryos at 24 hpf are displayed as N-24. RNA extracted from sham-injected and morpholino-injected embryos at 24 hpf are displayed as S-24 and M-24, respectively. RNA extracted from non-injected embryos at 36 hpf are displayed as N-36. RNA extracted from sham-injected and morpholino-injected embryos at 36 hpf are displayed as S-36 and M-36, respectively. RNA extracted from non-injected embryos at 48 hpf are displayed as N-48. RNA extracted from sham-injected and morpholino-injected embryos are displayed as S-48 and M-48, respectively. N=50.



**Figure 7. B-actin gel electrophoresis for *Danio rerio* zebrafish at 24, 36, and 48 hpf.** The DNA ladder is displayed as “L.” RNA extracted from non-injected embryos at 24 hpf are displayed as N-24. RNA extracted from sham-injected and morpholino-injected embryos at 24 hpf are displayed as S-24 and M-24, respectively. RNA extracted from non-injected embryos at 36 hpf are displayed as N-36. RNA extracted from sham-injected and morpholino-injected embryos at 36 hpf are displayed as S-36 and M-36, respectively. RNA extracted from non-injected embryos at 48 hpf are displayed as N-48. RNA extracted from sham-injected and morpholino-injected embryos are displayed as S-48 and M-48, respectively. N=50.



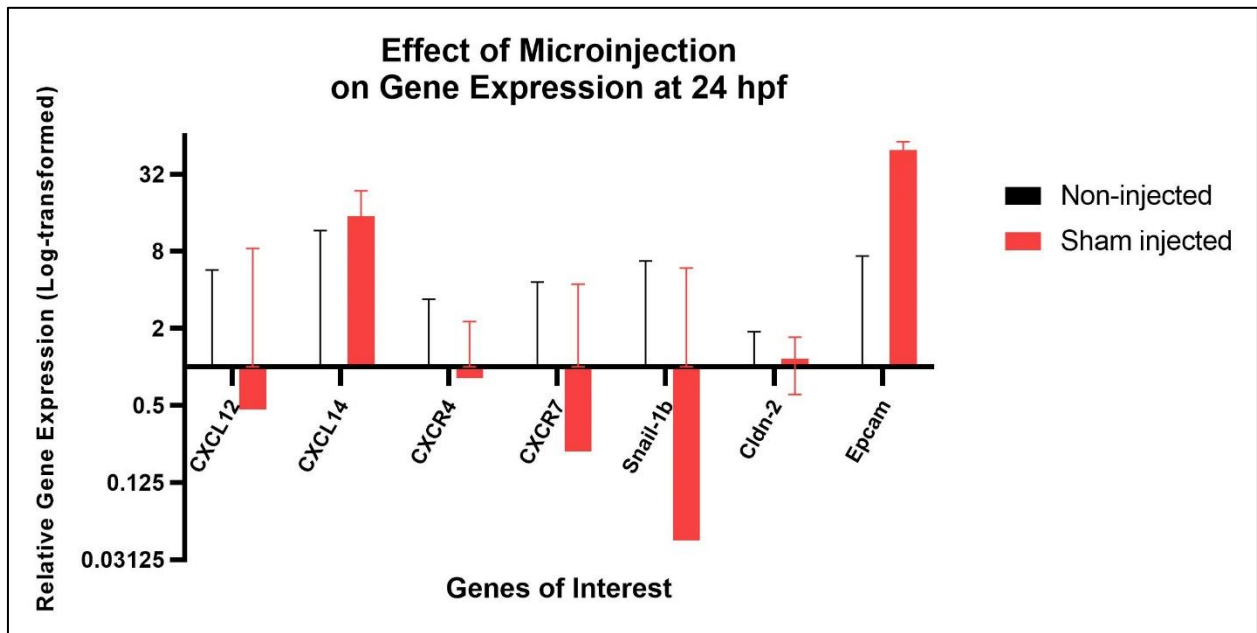
**Figure 8. CXCL12 gel electrophoresis for *Danio rerio* zebrafish at 24, 36, and 48 hpf.** The DNA ladder is displayed as “L.” RNA extracted from non-injected embryos at 24 hpf are displayed as N-24. RNA extracted from sham-injected and morpholino-injected embryos at 24 hpf are displayed as S-24 and M-24, respectively. RNA extracted from non-injected embryos at 36 hpf are displayed as N-36. RNA extracted from sham-injected and morpholino-injected embryos at 36 hpf are displayed as S-36 and M-36, respectively. RNA extracted from non-injected embryos at 48 hpf are displayed as N-48. RNA extracted from sham-injected and morpholino-injected embryos are displayed as S-48 and M-48, respectively. N=50.



**Figure 9. CXCL14 gel electrophoresis for *Danio rerio* zebrafish at 24, 36, and 48 hpf.** The DNA ladder is displayed as “L.” RNA extracted from non-injected embryos at 24 hpf are displayed as N-24. RNA extracted from sham-injected and morpholino-injected embryos at 24 hpf are displayed as S-24 and M-24, respectively. RNA extracted from non-injected embryos at 36 hpf are displayed as N-36. RNA extracted from sham-injected and morpholino-injected embryos at 36 hpf are displayed as S-36 and M-36, respectively. RNA extracted from non-injected embryos at 48 hpf are displayed as N-48. RNA extracted from sham-injected and morpholino-injected embryos are displayed as S-48 and M-48, respectively. N=50.

### 3.2. Quantifying the effect of microinjection on gene expression

The aim of this study was to examine the effects of CXCL14 knock-down on *Danio rerio* gene expression during lateral line development. In order to test our methodology, we first analyzed the relationship between microinjections and gene expression. Subsequently, zebrafish embryos were microinjected with 1 nl of phenol red buffer without morpholino (sham-injected). Microinjected embryos were compared to non-injected embryos at 24, 36, and 48 hpf. RT-qPCR was used to determine the mRNA levels of seven zebrafish genes expressed at various time points during lateral line development: *CXCL12*, *CXCL14*, *CXCR4*, *CXCR7*, *snail-1b*, *cldn-2*, and *epcam*. Relative gene expression levels were presented as Log<sub>2</sub> mRNA levels of sham-injected embryos relative to non-injected embryos.

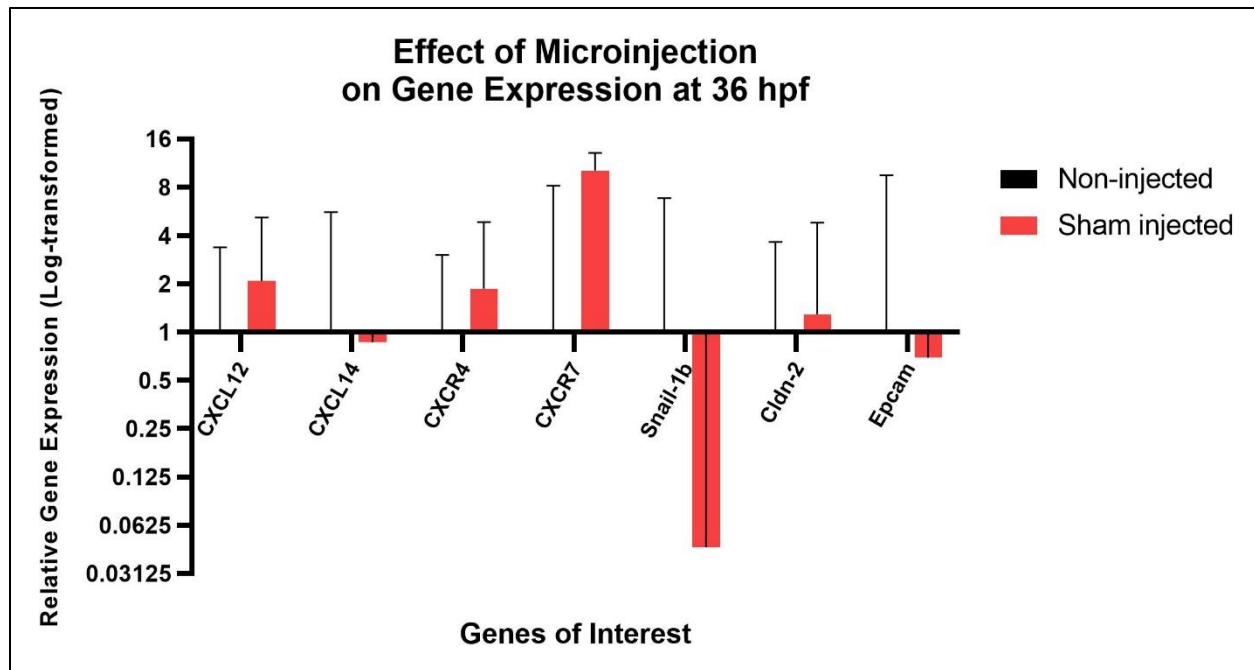


**Figure 10. Effect of Microinjection on Gene Expression in *Danio rerio* embryos at 24 hpf.** RT-qPCR examined the mRNA levels of seven zebrafish genes: *CXCL12*, *CXCL14*, *CXCR4*, *CXCR7*, *snail-1b*, *cldn-2*, and *epcam* expressed at 24 hours post-fertilization (hpf) following microinjection with 1 nl of phenol red. Relative gene expression levels were determined using the  $2^{-\Delta\Delta C_t}$  method with  $\beta$ -actin as the reference gene. The statistical significance of the data was determined by a two-tailed Mann-Whitney test between non-injected and sham-injected embryos at 24 hpf. Results were expressed as Log<sub>2</sub> mRNA fold-change values  $\pm$  S.D. (n=6), \*P  $\leq$  0.05.

Although the data were not statistically significant, it was observed that microinjection with phenol red caused a notable shift in gene expression at 24 hpf (Figure 10). When compared to the control (non-injected embryos), sham-injections resulted in a decrease in *CXCL12*, *CXCR7*, and *snail-1b* gene expression. More specifically, average mRNA levels for *CXCL12* decreased by 2.17-fold: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  4.734), sham-injection (n=6,  $\bar{x}$ = 2.17-fold change  $\pm$  7.979, p= 0.818). Moreover, average mRNA levels decreased by 4.6-fold for *CXCR7*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  3.609), sham-injection (n=6,  $\bar{x}$ = 4.6-fold change  $\pm$  4.226, p= 0.818) and by 22.7-fold for *snail-1b* following microinjection: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  5.731), sham-injected (n=6,  $\bar{x}$ = 22.7-fold change  $\pm$  5.902, p= 0.309).

Conversely, it was also observed that sham-injections caused an increase in gene expression at 24 hpf (Figure 10). When compared to the control (non-injected embryos), sham-injections resulted in an increase in *CXCL14* and *epcam* gene expression. Specifically, average mRNA levels for *CXCL14* increased by 15.2-fold: control (n= 6,  $\bar{x}$ = 0-fold change  $\pm$ 10.647), sham-injected (n=6,  $\bar{x}$ = 15.2-fold change  $\pm$  8.617, p= 0.24) and by 49.6-fold for *epcam* following microinjection: control (n= 6,  $\bar{x}$ = 0-fold change  $\pm$  6.346), sham-injected (n= 6,  $\bar{x}$ = 49.6-fold change  $\pm$  7.784, p= 0.179).

When compared to the control, however, *CXCR4* mRNA levels did not demonstrate a significant decrease in gene expression: control (n= 6,  $\bar{x}$ = 0-fold change  $\pm$  2.391), sham-injected embryos (n=6,  $\bar{x}$ = 1.22-fold change  $\pm$  1.446, p= 0.815). Similarly, *cldn-2* mRNA levels did not demonstrate a significant increase in gene expression following microinjection: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  6.346), sham-injected embryos (n=6,  $\bar{x}$ = 0.869-fold change  $\pm$  0.55, p= 1.159).



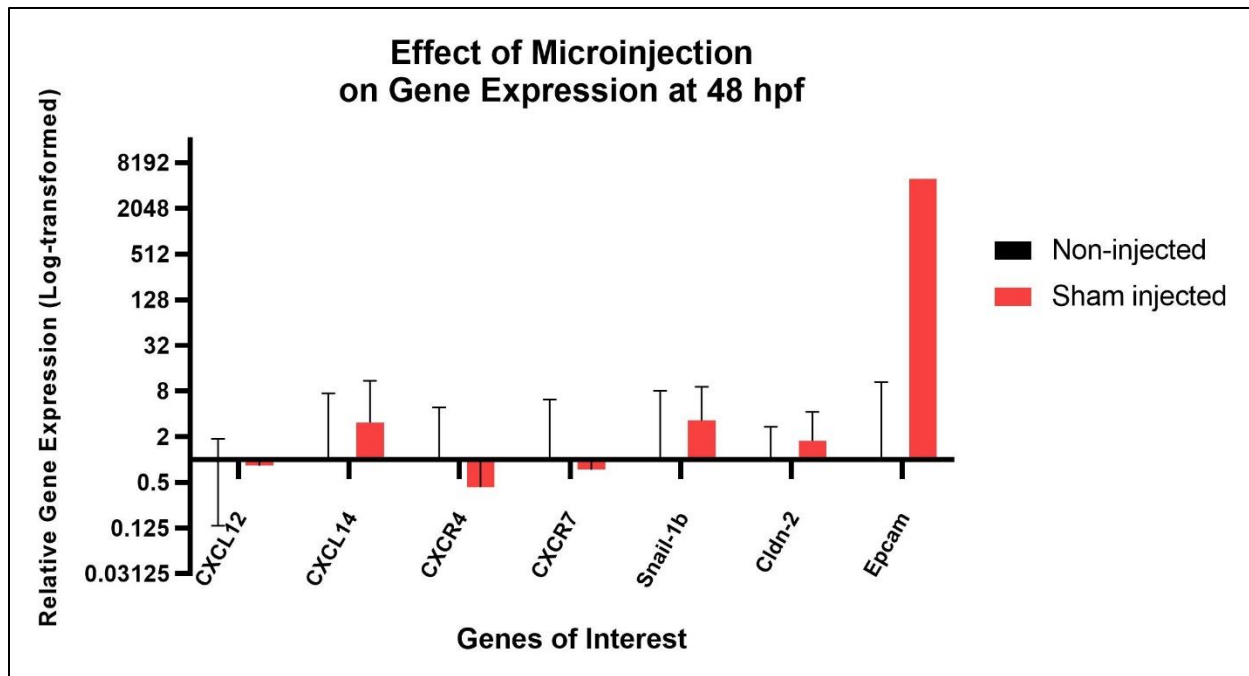
**Figure 11. Effect of Microinjection on Gene Expression in *Danio rerio* embryos at 36 hpf.** RT-qPCR examined the mRNA levels of seven zebrafish genes: *CXCL12*, *CXCL14*, *CXCR4*, *CXCR7*, *snail-1b*, *cldn-2*, and *epcam* expressed at 36 hours post-fertilization (hpf) following microinjection with 1 nl of phenol red. Relative gene expression levels were determined using the  $2^{-\Delta\Delta C_t}$  method with  $\beta$ -actin as the reference gene. The statistical significance of the data was determined by a two-tailed Mann-Whitney test between non-injected and sham-injected embryos at 36 hpf. Results are expressed as Log<sub>2</sub> fold-change values  $\pm$  S.D. (n=6), \*P $\leq$  0.05

Although the data were not statistically significant, it was observed that microinjection with phenol red caused a notable shift in gene expression at 36 hpf (Figure 11). When compared to the control (non-injected embryos), sham-injections resulted in increased gene expression for both *CXCL12* and *CXCR7*. Average mRNA levels for *CXCL12* increased by 2.1-fold (control: n=6,  $\bar{x}$ = 0-fold change  $\pm$  2.374; sham-injection: n=6,  $\bar{x}$ = 2.1-fold change  $\pm$  3.087, p= 0.177) whereas *CXCR7* gene expression increased by 10.1-fold (control: n=6,  $\bar{x}$ = 0-fold change  $\pm$  7.159; sham-injected: n=6,  $\bar{x}$ = 10.1-fold change  $\pm$  2.95, p= 0.428).

However, when compared to the control (non-injected embryos), *CXCR4* mRNA levels did not demonstrate a significant increase in gene expression: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$

2.029), sham-injection (n=6,  $\bar{x}$ = 1.86-fold change  $\pm$  2.986, p= 0.093). Likewise, *cldn-2* mRNA levels did not demonstrate a significant increase in gene expression following microinjection: control (n= 6,  $\bar{x}$ = 0-fold change  $\pm$  2.648), sham-injected (n= 6,  $\bar{x}$ = 1.29-fold change  $\pm$  3524, p= 0.121).

In contrast, it was also observed that sham-injections caused a decrease in *snail-1b* gene expression at 36 hpf: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  5.843), sham-injected (n=6,  $\bar{x}$ = 22-fold change  $\pm$  6.769, p= 0.818). Although similar trends were demonstrated, *CXCL14* and *epcam* mRNA levels did not demonstrate a significant decrease in gene expression when compared to the control (non-injected embryos). Specifically, average mRNA levels for *CXCL14* only decreased by 1.16-fold: control (n= 6,  $\bar{x}$ = 0-fold change  $\pm$  4.601), sham-injected (n=6,  $\bar{x}$ = 1.16-fold change  $\pm$  8.989, p= 0.428). Similarly, *epcam* gene expression only decreased by 1.44-fold: control (n= 6,  $\bar{x}$ = 0-fold change  $\pm$  8.512), sham-injected (n= 6,  $\bar{x}$ = 1.44-fold change  $\pm$  7.874, p= 0.699).



**Figure 12. Effect of Microinjection on Gene Expression in *Danio rerio* embryos at 48 hpf.** RT-qPCR examined the mRNA levels of seven zebrafish genes: *CXCL12*, *CXCL14*, *CXCR4*, *CXCR7*, *snail-1b*, *cldn-2*, and *epcam* expressed at 48 hours post-fertilization (hpf) following microinjection with 1 nl of phenol red. Relative gene expression levels were determined using the  $2^{-\Delta\Delta C_t}$  method with  $\beta$ -actin as the reference gene. The statistical significance of the data was determined by a two-tailed Mann-Whitney test between non-injected and sham-injected embryos at 48 hpf. Results are expressed as Log<sub>2</sub> fold-change values  $\pm$  S.D. (n=6), \*P $\leq$  0.05.

Although the data were not statistically significant, it was observed that microinjection with phenol red caused a notable shift in gene expression at 48 hpf (Figure 12). When compared to the control (non-injected embryos), sham-injections resulted in an increase in gene expression for *CXCL14*, *snail-1b*, and *epcam*. More specifically, average mRNA levels for *CXCL14* increased by 3.06-fold (control: n=6,  $\bar{x}$ = 0-fold change  $\pm$  6.39), sham-injected (control: n=6,  $\bar{x}$ = 3.06-fold change  $\pm$  7.797, p= 0.064). Moreover, following microinjection, average mRNA levels for *snail-1b* increased by 3.25-fold: control (n= 6,  $\bar{x}$ = 0-fold change  $\pm$  7.05), sham-injected (n=6,  $\bar{x}$ = 3.25-fold change  $\pm$  5.782, p= 0.937) and by 4963-fold for *epcam*: control (n= 6,  $\bar{x}$ = 0-fold change  $\pm$  9.414), sham-injected (n= 6,  $\bar{x}$ = 4963-fold change  $\pm$  9.851, p= 0.309). When compared



to the control (non-injected embryos), however, *cldn-2* mRNA levels did not demonstrate a significant increase in gene expression: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  1.699), sham-injected (n=6,  $\bar{x}$ = 1.75-fold change  $\pm$  2.465, p= 0.66).

Conversely, it was also observed that sham-injections caused a decrease in *CXCR4* gene expression: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$ 3.868), sham-injected (n=6,  $\bar{x}$ = 2.33-fold change  $\pm$  2.192, p= 0.699). Although similar trends were demonstrated, *CXCL12* and *CXCR7* mRNA levels did not demonstrate a significant decrease in gene expression when compared to the control (non-injected embryos). Specifically, average mRNA levels for *CXCL12* only decreased by 1.2-fold: control (n= 6,  $\bar{x}$ = 0-fold change  $\pm$  0.866), sham-injected (n=6,  $\bar{x}$ = 1.2-fold change  $\pm$  5.100, p= 0.536) and by 1.36-fold for *CXCR7*: control (n= 6,  $\bar{x}$ = 0-fold change  $\pm$  5.172), sham-injected (n= 6,  $\bar{x}$ = 1.36-fold change  $\pm$  5.337, p= 0.393).

<b>Summary of mRNA fold-change values relative to non-injected embryos</b>			
	<b>24 hpf</b>	<b>36 hpf</b>	<b>48 hpf</b>
<b><i>CXCL12</i></b>	Decrease in gene expression by 2.17-fold $\pm$ 7.979. p= 0.818	Increase in gene expression by 2.1-fold $\pm$ 3.08. p= 0.177	Decrease in gene expression by 1.2-fold $\pm$ 5.100. p= 0.536
<b><i>CXCL14</i></b>	Increase in gene expression by 15.2-fold $\pm$ 8.617. p= 0.24	Decrease in gene expression by 1.16-fold $\pm$ 8.989. p= 0.428	Increase in gene expression by 3.06-fold $\pm$ 7.797. p= 0.064
<b><i>CXCR4</i></b>	Decrease in gene expression by 1.22-fold $\pm$ 1.446. p= 0.815	Increase in gene expression by 1.86-fold $\pm$ 2.986. p= 0.093	Decrease in gene expression by 2.33-fold $\pm$ 2.192. p= 0.699
<b><i>CXCR7</i></b>	Decrease in gene expression by 4.6-fold $\pm$ 4.226. p= 0.818	Increase in gene expression by 10.1-fold $\pm$ 2.95. p= 0.428	Decrease in gene expression by 1.36-fold $\pm$ 5.337. p= 0.393
<b><i>Snail-1b</i></b>	Decrease in gene expression by 22.7-fold $\pm$ 5.902. p= 0.309	Decrease in gene expression by 22-fold $\pm$ 6.769. p= 0.818	Increase in gene expression by 3.25-fold $\pm$ 5.782. p= 0.937
<b><i>Cldn-2</i></b>	Increase in gene expression by 0.869-fold $\pm$ 0.55. p= 1.159	Increase in gene expression by 1.29-fold $\pm$ 3524. p= 0.121	Increase in gene expression by 1.75-fold $\pm$ 2.465. p= 0.66
<b><i>Epcam</i></b>	Increase in gene expression by 49.6-fold $\pm$ 7.784. p= 0.179	Decrease in gene expression by 1.44-fold $\pm$ 7.874. p= 0.699	Increase in gene expression by 4963-fold $\pm$ 9.851. p= 0.309

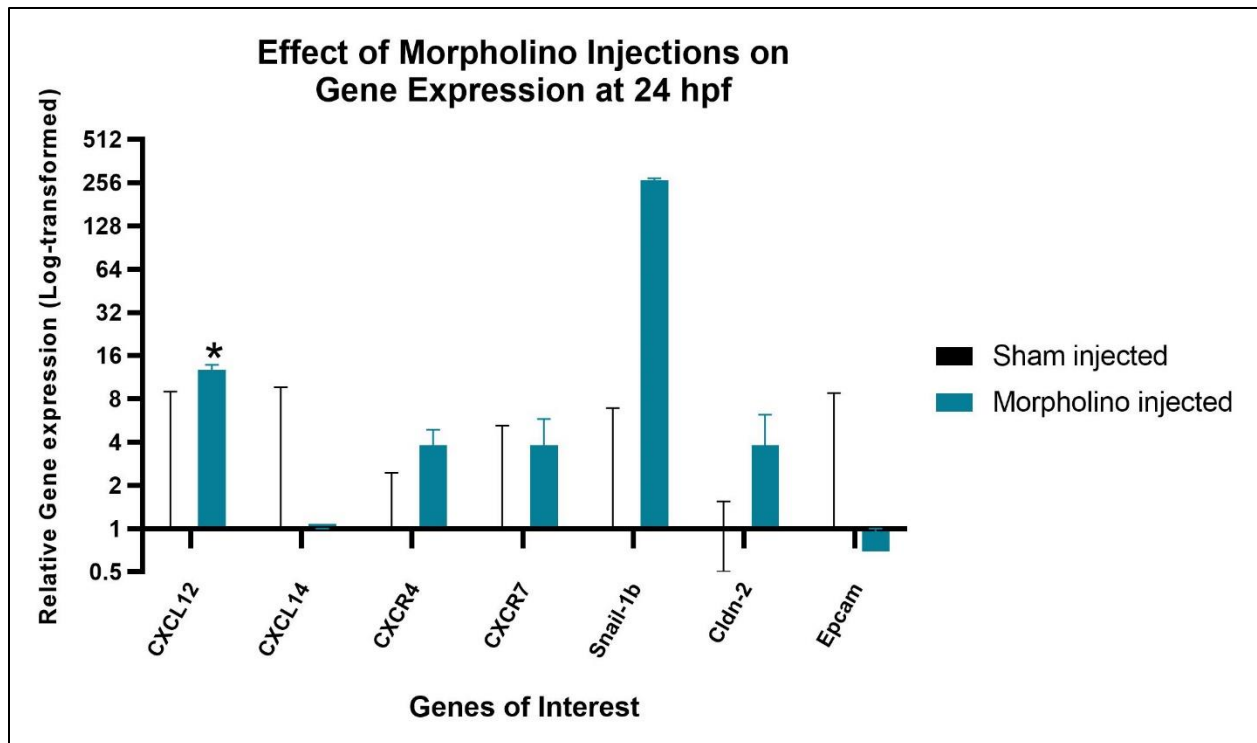
**Table 3. Summary of mRNA fold-change values for sham-injected embryos relative to non-injected embryos.** Data were presented as fold-change in gene expression  $\pm$  S.D. \*P  $\leq$  0.05.

It is interesting to note the impact microinjections have on gene expression. Although none of the data were statistically significant, there is evidence to support that microinjections can affect gene expression (Table 3). Given sham-injection's ability to interfere with gene expression, we decided to compare data from morpholino-injected embryos relative to sham-

injected embryos. This allowed us the opportunity to analyze the difference in injection contents and their respective impact on gene expression.

### 3.3. *Quantifying the effect of morpholino injection on gene expression*

Upon analyzing the effects of microinjection on gene expression, we examined the relationship between CXCL14 knock-down and *Danio rerio* gene expression during lateral line development. As a result, zebrafish embryos were microinjected with 1 nl of CXCL14 antisense morpholino in phenol red buffer. Morpholino-injected embryos were compared to sham-injected embryos at 24, 36, and 48 hpf. RT-qPCR was used to determine the mRNA levels of seven zebrafish genes expressed at various time points during lateral line development: *CXCL12*, *CXCL14*, *CXCR4*, *CXCR7*, *snail-1b*, *cldn-2*, and *epcam*. Relative gene expression levels were presented as Log<sub>2</sub> mRNA levels of morpholino-injected embryos relative to sham-injected embryos.

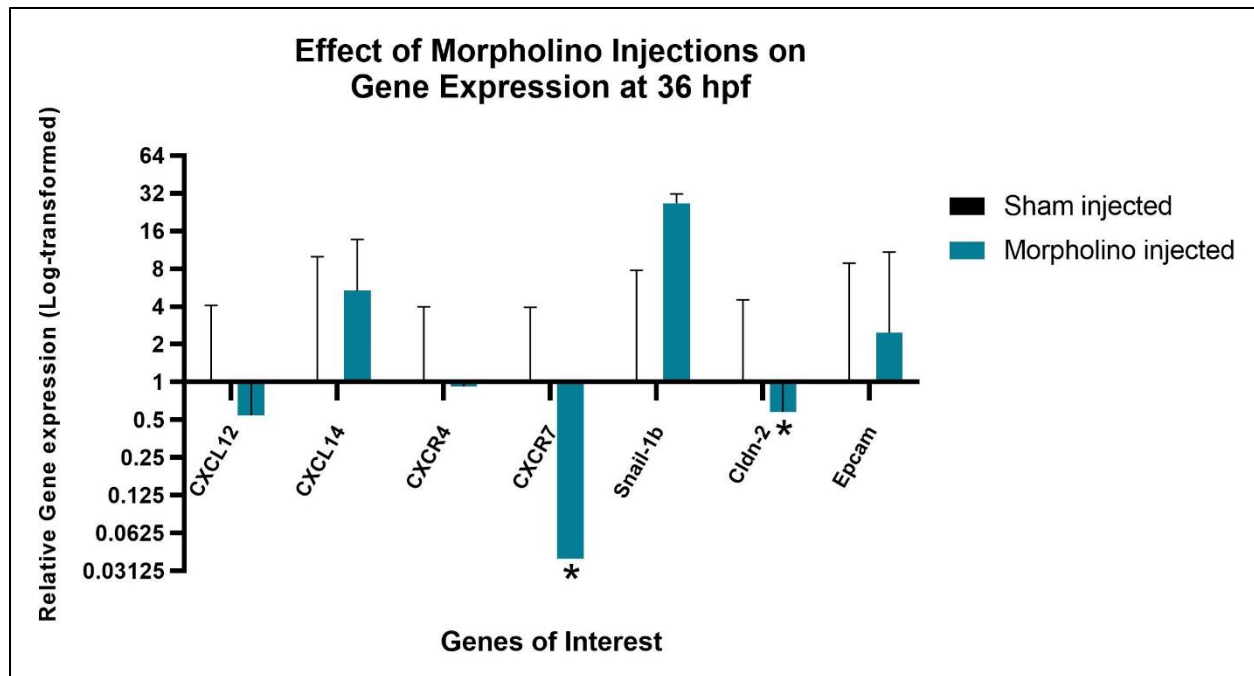


**Figure 13. Effect of Morpholino injections on Gene Expression in *Danio rerio* embryos at 24 hpf.** RT-qPCR examined the mRNA levels of seven zebrafish genes: *CXCL12*, *CXCL14*, *CXCR4*, *CXCR7*, *snail-1b*, *cldn-2*, and *epcam* expressed at 24 hours post-fertilization (hpf) following microinjection with either 1 nl of phenol red (sham) or CXCL14 antisense morpholino in phenol red buffer. Relative gene expression levels were determined using the  $2^{-\Delta\Delta C_t}$  method with  $\beta$ -actin as the reference gene. The statistical significance of the data was determined by a two-tailed Mann-Whitney test between sham-injected embryos and morpholino-injected embryos at 24 hpf. Results were expressed as Log2 fold-change values  $\pm$  S.D. (n=6), \*P $\leq$  0.05

When compared to the control (sham-injected embryos), it was observed that microinjection with CXCL14 antisense morpholino caused a notable increase in gene expression at 24 hpf for five out of the seven genes: *CXCL12*, *CXCR4*, *CXCR7*, *snail-1b*, and *cldn-2* (Figure 13). After microinjection with CXCL14 antisense morpholino, average mRNA levels increased by 3.78-fold for *CXCR4*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  1.446), morpholino-injection (n=6,  $\bar{x}$ = 3.78-fold change  $\pm$  1.09, p= 0.309), by 3.78-fold for *CXCR7*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  4.226), morpholino-injected (n=6,  $\bar{x}$ = 3.78-fold change  $\pm$  2.023, p= 0.937), and by 267-fold for

*snail-1b*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  5.902), morpholino-injected (n=6,  $\bar{x}$ = 267-fold change  $\pm$  6.823, p= 0.179). In particular, *CXCL12* gene expression following morpholino-injections were statistically significant from embryos receiving sham-injections. In comparison to controls, average mRNA levels for *CXCL12* increased by 12.6-fold: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  7.979), morpholino-injection (n=6,  $\bar{x}$ = 12.6-fold change  $\pm$  1.147, \*p= 0.026). Moreover, *cldn-2* gene expression following morpholino-injections demonstrated a 3.78-fold increase with a modest p-value of 0.056: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  0.550), morpholino-injected (n=6,  $\bar{x}$ = 3.78-fold change  $\pm$  2.434, p= 0.056).

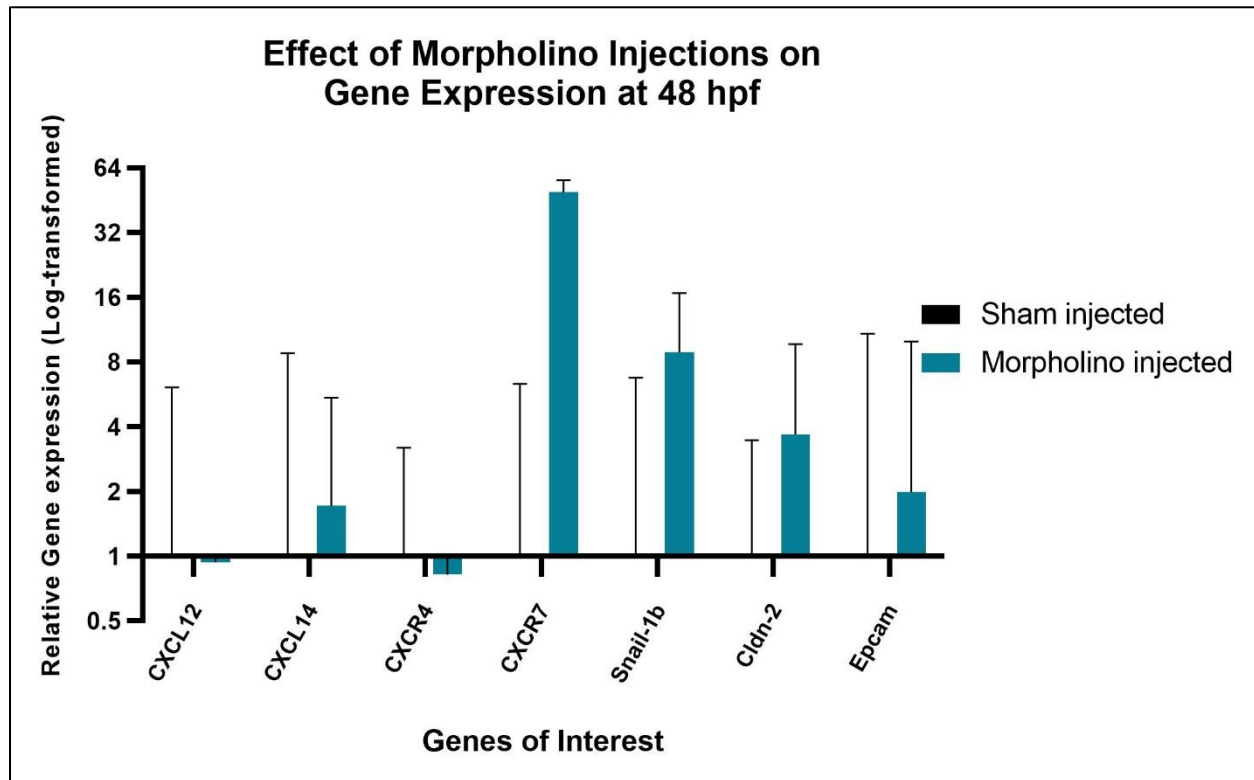
Compared to the control (sham-injected embryos), *CXCL14* and *epcam* mRNA levels did not demonstrate a significant change in gene expression. Following morpholino-injections, *CXCL14* only demonstrated a 0.99-fold increase in gene expression: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  8.617), morpholino-injected (n=6,  $\bar{x}$ = 0.99-fold change  $\pm$  3.596, p= 0.588). Likewise, *epcam* mRNA levels only decreased by 1.44-fold following morpholino-injections: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  7.784), morpholino-injected (n= 6,  $\bar{x}$ = 1.44-fold change  $\pm$  8.23, p= 0.699).



**Figure 14. Effect of Morpholino injections on Gene Expression in *Danio rerio* embryos at 36 hpf.** RT-qPCR examined the mRNA levels of seven zebrafish genes: *CXCL12*, *CXCL14*, *CXCR4*, *CXCR7*, *snail-1b*, *cldn-2*, and *epcam* expressed at 36 hours post-fertilization (hpf) following microinjection with either 1 nl of phenol red (sham) or CXCL14 antisense morpholino in phenol red buffer. Relative gene expression levels were determined using the  $2^{-\Delta\Delta C_t}$  method with  $\beta$ -actin as the reference gene. The statistical significance of the data was determined by a two-tailed Mann-Whitney test between sham-injected embryos and morpholino-injected embryos at 36 hpf. Results were expressed as Log2 fold-change values  $\pm$  S.D. (n=6), \*P $\leq$  0.05

When compared to the control (sham-injected embryos), it was observed that microinjection with CXCL14 antisense morpholino caused a notable increase in gene expression at 36 hpf (Figure 14). More specifically, average mRNA levels increased by 5.37-fold for *CXCL14*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  8.989), morpholino-injection (n=6,  $\bar{x}$ = 5.37-fold change  $\pm$  8.342, p= 0.792), by 26.6-fold for *snail-1b*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  6.769), morpholino-injected (n=6,  $\bar{x}$ = 26.6-fold change  $\pm$  4.981, p= 0.699), and by 2.47-fold for *epcam*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  7.874), morpholino-injected (n=6,  $\bar{x}$ = 2.47-fold change  $\pm$  8.403, p= 0.309).

In contrast, it was also observed that mRNA levels decreased following morpholino-injections. In particular, *CXCR7* and *cldn-2* gene expression for morpholino-injected embryos were statistically significant from embryos receiving sham-injections. When compared to the control, average mRNA levels decreased by 25.6-fold for *CXCR7*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  2.95), morpholino-injected (n=6,  $\bar{x}$ = 25.6-fold change  $\pm$  0, \*p= 0.017) and by 1.75-fold for *cldn-2*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  3.524), morpholino-injected (n=6,  $\bar{x}$ = 1.75-fold change  $\pm$  2.658, \*p= 0.036). Although similar trends were demonstrated, mRNA levels for both *CXCL12* and *CXCR4* only decreased by less than 2-fold. Following morpholino-injections, gene expression decreased by 1.85-fold for *CXCL12*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  3.087), morpholino-injected: (n=6,  $\bar{x}$ = 1.85-fold change  $\pm$  1.993, p= 0.930) and by 1.08-fold for *CXCR4*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  2.986), morpholino-injected: (n=6,  $\bar{x}$ = 1.08-fold change  $\pm$  2.36, p= 0.179).



**Figure 15. Effect of Morpholino injections on Gene Expression in *Danio rerio* embryos at 48 hpf.** RT-qPCR examined the mRNA levels of seven zebrafish genes: *CXCL12*, *CXCL14*, *CXCR4*, *CXCR7*, *snail-1b*, *cldn-2*, and *epcam* expressed at 48 hours post-fertilization (hpf) following microinjection with either 1 nl of phenol red (sham) or CXCL14 antisense morpholino in phenol red. Relative gene expression levels were determined using the  $2^{-\Delta\Delta C_t}$  method with  $\beta$ -actin as the reference gene. The statistical significance of the data was determined by a two-tailed Mann-Whitney test between non-injected embryos and either sham-injected or morpholino-injected embryos at 48 hpf. Results were expressed as Log<sub>2</sub> fold-change values  $\pm$  S.D. (n=6), \*P $\leq$  0.05

When compared to the control (sham-injected embryos), it was observed that microinjection with CXCL14 antisense morpholino caused a notable increase in gene expression at 48 hpf (Figure 15). More specifically, average mRNA levels increased by 49.3-fold for *CXCR7*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  5.337), morpholino-injected: (n=6,  $\bar{x}$ = 49.3-fold change  $\pm$  6.655, p= 0.179), by 8.9-fold for *snail-1b*: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  5.782), morpholino-injected: (n=6,  $\bar{x}$ = 8.9-fold change  $\pm$  7.866, p= 0.484), and by 3.68-fold for *cldn-2*:



control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  2.465), morpholino-injected (n=6,  $\bar{x}$ = 3.68-fold change  $\pm$  6.014, p= 0.937). Although similar trends were demonstrated, average mRNA levels for *CXCL14* only increased by 1.71-fold: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  7.797), morpholino-injected (n=6,  $\bar{x}$ = 1.71-fold change  $\pm$  3.74, p= 0.662). Similarly, when compared to the control, average mRNA levels for *epcam* only increased by 1.99-fold: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  9.851), morpholino-injected (n=6,  $\bar{x}$ = 1.99-fold change  $\pm$  7.986, p= 0.484).

In contrast, it was also observed that mRNA levels were not affected by morpholino-injections at 48 hpf. In particular, *CXCL12* and *CXCR4* average mRNA levels experienced a small decrease in expression (< 2-fold). More specifically, when compared to the control (sham-injected embryos), *CXCL12* gene expression only decreased by 1.07-fold: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  5.100), morpholino-injected (n=6,  $\bar{x}$ = 1.07-fold change  $\pm$  2.083, p= 0.547). Likewise, following morpholino-injections, *CXCR4* mRNA levels only decreased by 1.21-fold: control (n=6,  $\bar{x}$ = 0-fold change  $\pm$  2.192), morpholino-injected (n=6,  $\bar{x}$ = 1.21-fold change  $\pm$  2.408, p= 0.309).

<b>Summary of mRNA fold-change values relative to sham-injected embryos</b>			
	<b>24 hpf</b>	<b>36 hpf</b>	<b>48 hpf</b>
<b><i>CXCL12</i></b>	Increase in gene expression by 12.6-fold $\pm$ 1.147. *p= 0.026	Decrease in gene expression by 1.85-fold $\pm$ 1.993. p= 0.930	Decrease in gene expression by 1.07-fold $\pm$ 2.083. p= 0.547
<b><i>CXCL14</i></b>	Increase in gene expression by 0.99-fold $\pm$ 3.596. p= 0.588	Increase in gene expression by 5.37-fold $\pm$ 8.342. p= 0.792	Increase in gene expression by 1.71-fold $\pm$ 3.74. p= 0.662
<b><i>CXCR4</i></b>	Increase in gene expression by 3.78-fold $\pm$ 1.09. p= 0.309	Decrease in gene expression by 1.08-fold $\pm$ 2.36. p= 0.179	Decrease in gene expression by 1.21-fold $\pm$ 2.408. p= 0.309
<b><i>CXCR7</i></b>	Increase in gene expression by 3.78-fold $\pm$ 2.023. p= 0.937	Decrease in gene expression by 25.6-fold $\pm$ 0. *p= 0.017	Increase in gene expression by 49.3-fold $\pm$ 6.655. p= 0.179
<b><i>Snail-1b</i></b>	Increase in gene expression by 267-fold $\pm$ 6.823. p= 0.179	Increase in gene expression by 26.6-fold $\pm$ 4.981. p= 0.699	Increase in gene expression by 8.9-fold $\pm$ 7.866. p= 0.484
<b><i>Cldn-2</i></b>	Increase in gene expression by 3.78-fold $\pm$ 2.434. p= 0.056	Decrease in gene expression by 1.75-fold $\pm$ 2.658. *p= 0.036	Increase in gene expression by 3.68-fold $\pm$ 6.014. p= 0.937
<b><i>Epcam</i></b>	Decrease in gene expression by 1.44-fold $\pm$ 8.23. p= 0.699	Increase in gene expression by 2.47-fold $\pm$ 8.403. p= 0.309	Increase in gene expression by 1.99-fold $\pm$ 7.986. p= 0.484

**Table 4. Summary of mRNA fold-change values for CXCL14 antisense morpholino-injected embryos relative to sham-injected embryos.** Data were presented as fold-change in gene expression  $\pm$  S.D. \*P  $\leq$  0.05.

## DISCUSSION

The aim of this study was to examine the effects of CXCL14 knock-down on *Danio rerio* gene expression during lateral line development. Zebrafish embryos at the 1-cell stage were microinjected with either 1 nl of phenol red or CXCL14 antisense morpholino in phenol red buffer. Microinjected embryos were then incubated at 24, 36, and 48 hpf and compared against non-injected embryos. To determine the effects of microinjection on gene expression, RT-qPCR was used to determine the mRNA levels of seven zebrafish genes during lateral line development at 24, 36, and 48 hpf: *CXCL12*, *CXCL14*, *CXCR4*, *CXCR7*, *snail-1b*, *cldn-2*, and *epcam*. To compare the effects of microinjection on gene expression, relative gene expression levels of sham-injected embryos were compared to non-injected embryo mRNA levels at 24, 36, and 48 hpf (Figures 10, 11, and 12). The data were presented as Log<sub>2</sub> mRNA levels of injected embryos relative to non-injected embryos. To compare the effects of morpholino-injections on gene expression, relative gene expression levels of morpholino-injected embryos were compared to sham-injected embryo mRNA levels at 24, 36, and 48 hpf (Figures 13, 14, and 15). The data were presented as Log<sub>2</sub> mRNA levels of injected embryos relative to sham-injected embryos.

### 4.1. *CXCL14* modulating the *CXCL12*-*CXCR4* signaling pathway

Although the data were not all statistically significant, it can be noted that both sham- and morpholino-injections influence gene expression. When comparing *CXCL12* gene expression between sham- and morpholino-injections, there is a distinct inverse relationship between the two. After sham-injections, *CXCL12* gene expression decreased by 2.17-fold at 24 hpf, followed by 2.1-fold increase at 36 hpf (Figures 10 and 11) whereas after morpholino-injections *CXCL12* gene expression increased by 12.6-fold at 24 hpf before decreasing by 1.8-fold at 36 hpf (Figures

13 and 14). The inverse change in *CXCL12* gene expression after *CXCL14* knockdown indicates that *CXCL14* is modulating *CXCL12* gene expression. These findings coincide with other studies examining *CXCL14* function relative to *CXCL12* activity (Tanegashima and Hara, 2012; Tanegashima et. al, 2013).

A similar trend is also observed with expression of *CXCL12*'s receptors: *CXCR4* and *CXCR7*. Following sham-injections, *CXCR4* gene expression decreased by 1.2-fold at 24 hpf, followed by a 1.8-fold increase at 36 hpf (Figures 10 and 11). Inversely, morpholino-injected embryos had an increase in *CXCR4* gene expression by 2.7-fold at 24 hpf, followed by a 1.08-fold decrease in gene expression at 36 hpf (Figures 13 and 14). This relationship can also be seen for *CXCR7* gene expression. Following sham-injections, *CXCR7* mRNA levels decreased by 4.6-fold at 24 hpf before increasing by 10.1-fold at 36 hpf (Figures 10 and 11). Whereas after *CXCL14* morpholino-injections *CXCR7* gene expression increased by 3.78-fold at 24 hpf, then decreased by 25.6-fold at 36 hpf (Figures 13 and 14). As with *CXCL12*, this result suggests *CXCL14* play a role in modulating *CXCR4* and *CXCR7* mRNA levels. These findings coincide with previous studies characterizing *CXCR4* and *CXCR7* as receptors for *CXCL12* (Tanegashima and Hara, 2012; Tanegashima et. al, 2013).

Additionally, a notable trend is found between injections for *CXCL14*. Following sham-injections, *CXCL14* increased gene expression by 15-fold at 24 hpf, followed by a 1.16-fold decrease at 36 hpf and later a 3-fold increase at 48 hpf (Figures 10, 11, and 12). More notably, these results have an inverse relationship with *CXCL12* sham-injection results. This suggests the idea that *CXCL14* and *CXCL12* gene expression modulate each other.

In addition to *CXCL14* modulating *CXCL12*-*CXCR4* and *CXCR7* gene expression, we have observed a significant change in *snail-1b* gene expression following *CXCL14* knockdown.

During sham-injections, *snail-1b* gene expression steadily increased between 24 hpf and 48 hpf (Figures 10, 11, and 12). Following treatment with morpholino-injections, *snail-1b* gene expression decreased by substantial amounts (Figures 13, 14, and 15). Snail 1-b is typically found in the leading cells of the lateral line system during development. Our results suggest CXCL14's capability to impact lateral line development outside the CXCL12-CXCR4 signaling pathway.

#### 4.2. Future Research

This research project could be expanded in several different ways in order to inform our understanding of the relationship between CXCL14 modulation of the CXCL12-CXCR4 signaling pathway and lateral line development. Future studies include a continuation of qPCR trials. By running more qPCRs, we can definitively support our claim that CXCL14 is modulating the CXCL12-CXCR4 signaling pathway. It will also be important to visualize changes in gene expression in morpholino injected embryos. Through *in-situ* hybridization, we can confirm qPCR results by visualizing increased or decreased gene expression of CXCL12, CXCR4, and CXCR7 in the lateral line tissues. This is important because these genes may be expressed in other tissues in the embryos, not just the lateral line system. In addition to *in-situ* hybridization, we should examine the morphology of the neuromasts' cells and sensory neuron innervation in sham and morpholino injected embryos to determine if *CXCL14* knockdown affects the formation or innervation of neuromasts. Beyond morphological changes, functional changes should be examined. For example, a flow-chamber assay may help provide deeper insight on the functional changes occurring after *CXCL14*-knockdown. By allowing morpholino-injected embryos to develop into further stages, we can assess their ability to orient themselves in moving water. By analyzing their ability to swim in the event of any water-current stimuli, we

can determine whether *CXCL14* knockdown functionally affects lateral line development in zebrafish.

In conclusion, our study has provided evidence supporting *CXCL14* modulation of *CXCL12* gene expression. Additionally, we provide evidence that *CXCL14* modulates both *CXCR4* and *CXCR7* gene expression, thereby suggesting its ability to modulate the *CXCL12*-*CXCR4* signaling pathway more broadly. Based on our overall findings, we suggest that *CXCL14* may impact lateral line development. However, further work needs to be done to understand the full extent of these relationships.

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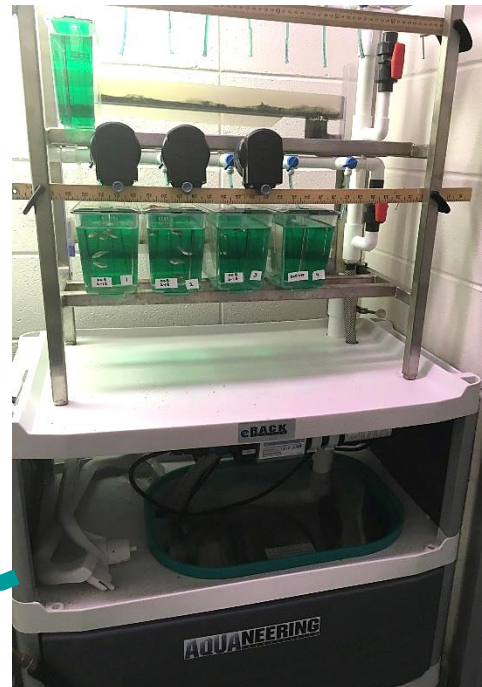
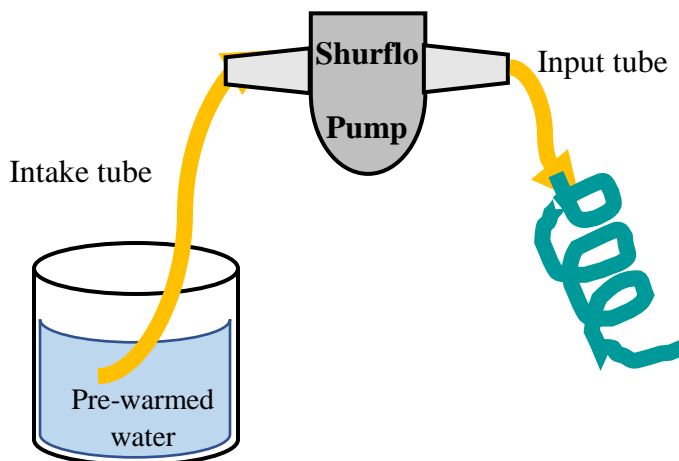
## APPENDIX

### Appendix I: Zebrafish Husbandry

#### How to Add Water to the Fish System

\* Make sure aged tap water is pre-warmed (28.5°C) in bucket(s) in the Fish Room before starting

1. Connect the input rubber tube to the output port on the filtration pump (Pentair- Aquatic Habitats, Minneapolis, MN, USA)
2. Connect one end of the intake tube to the input port on the filtration pump
  - a. The other end of the intake tube goes into the bucket with pre-warmed aged tap water
3. Lift the clear plastic cover of the “sump” from the fish system (Aquanearing, San Diego, CA, USA) to check water levels
4. Turn on the pump and add water until water level reaches the bottom of the white horizontal drain tube in the “sump” area of the fish system
5. Turn off the pump, remove input and output tubes, and replace the clear plastic cover of the sump
6. Refill water buckets with water from the stainless-steel tanks in the aquarium room so there will be pre-warmed water for the next day

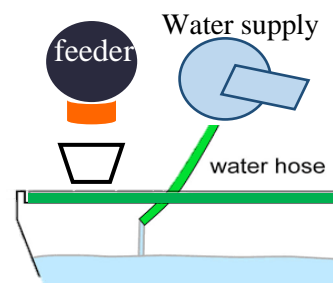
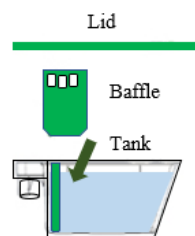


## How to Clean Tanks

1. Lift the tank and allow water to flow down the drain tube
2. Place the tank on the counter near the sink

Replace with a new tank

- a. Insert the green baffle and fill the new tank with water from the water supply
  - b. Use a net to gather fish and place them inside the fresh, clean tank
    - i. Place the tank in the corresponding spot- ensure zebrafish birthdates and tank number are displayed on the front of the tank
  - c. Ensure the “hose” is in the second hole of the lid
  - d. Place the funnel in the first hole of the lid
  - e. Place the food feeder above the funnel and screw tightly
    - i. Ensure the feeder is aligned with the funnel so that the food falls through the funnel and into the tank
3. Dump the water from the dirty tank down the drain
  4. Rinse the tank with tap water
    - a. Pour bleach into the tank
      - i. Enough to barely fill the bottom of the tank  
1 mL of bleach for every 50 mL of H<sub>2</sub>O
  5. Fill the tank with water
    - a. Careful not to fill the tank all the way since it needs to be moved from the counter and onto the shelf
      - i. **DO NOT SPILL THIS WATER INTO ANY OF THE BUCKETS**
  6. Carefully place the tank on the back of the bottom shelf
  7. Fill the rest of the tank with water using a plastic cup
    - a. You want the bleach to reach the overhang
      - i. The tank is in the far back so that the bleach water from the overhang can run down the back of the wall and into the drain
  8. Rinse the lid and baffle with dI water and place it in the tank
  9. Allow the tank to be submerged in bleach water for at least 24 hours
  10. After the 24 hours have passed, rinse the tank and its parts with tap water, then rinse them with dI water at least 3 times
  11. Leave items on the shelf to air dry



## Appendix II: Preparing Solutions

### How to make 1x Danieau solution

1. Gather reagents to make 1x Danieau solution

Stock Concentration	Concentration needed
5 M	58 mM NaCl
3 M	0.7 mM KCl
100 mM	0.4 mM MgSO <sub>4</sub>
100 mM	0.6 mM Ca (NO <sub>3</sub> ) <sub>2</sub>
0.5 M	5 mM HEPES pH 7.6

2. Calculate the amounts of reagents needed to make 5 mL of 1x Danieau solution

1 M= 1000 mM

1 mL= 1000 ul

Stock [concentration] x stock volume = Final [concentration] x final volume (5 mL)

- NaCl

$$5000 \text{ mM} (x) = 58 \text{ mM} (5 \text{ mL}) \rightarrow x = (290 \text{ mM/mL}) / 5000 \text{ mM} \rightarrow 0.058 \text{ mL or } 58 \text{ ul}$$

- KCl

$$3000 \text{ mM} (x) = 0.7 \text{ mM} (5 \text{ mL}) \rightarrow x = (3.5 \text{ mM/mL}) / 3000 \text{ mM} \rightarrow 0.0011 \text{ mL or } 1.166 \text{ ul}$$

or 11.66 ul from a 1:10 dilution

- MgSO<sub>4</sub>

$$100 \text{ mM} (x) = 0.4 \text{ mM} (5 \text{ mL}) \rightarrow x = (2 \text{ mM/mL}) / 100 \text{ mM} \rightarrow 0.02 \text{ mL} \rightarrow 20 \text{ ul}$$

- Ca (NO<sub>3</sub>)<sub>2</sub>

$$100 \text{ mM} (x) = 0.6 \text{ mM} (5 \text{ mL}) \rightarrow x = (3 \text{ mM/mL}) / 100 \text{ mM} \rightarrow 0.03 \text{ mL} \rightarrow 30 \text{ ul}$$

- HEPES

$$500 \text{ mM} (x) = 5 \text{ mM} (5 \text{ mL}) \rightarrow x = (25 \text{ mM/mL}) / 500 \text{ mM} \rightarrow 0.05 \text{ mL} \rightarrow 50 \text{ ul}$$

**Total: 169.66 ul**

- Milli Q water

$$5 \text{ mL} \rightarrow 5000 \text{ ul} - 169.66 \text{ ul reagents} = 4830.34 \text{ ul Milli Q}$$

## How to Resuspend Morpholino

1. Find morpholino (Gene Tools, LLC., Philomath, OR, USA)
  - a. Stock solution of SCYBA 2: 300 nmol freeze dried
  - b. Sequence: 5'- CCGTACTACAGCGATTCATCCCCAA-3'
2. Resuspend morpholino in 250 ul of 1x Danieau solution
  - a. DO NOT VORTEX OR SHAKE VIAL
    - i. Swirl the solution by slowing turning the vial around the sides, then up and down
      1. This ensures that the 1x Danieau solution reaches the freeze-dried powder on the cap and around the vial
3. Parafilm the morpholino vial and let it sit in the fridge for roughly 30 minutes
4. Aliquot the morpholino and label. Store stock solution and aliquots in “Scyba MO box” in -20 °C

Number of Aliquots	Amount
1	100 ul aliquot (remains in vial)
1	50 ul aliquot
1	25 ul aliquot
15	5 ul aliquot

5. When microinjecting morpholino, make sure it is 1 nL in volume
  - a. Between two lines in width (0.2 mm in width)

## Morpholino Troubleshooting Guide

1. Accidentally pipetted 1,100 ul of Danieau solution, making morpholino 4x too dilute
    - a. Decided to lyophilize morpholino
      - i. Placed morpholino in -80 °C freezer
      - ii. When frozen, put morpholino on counter and parafilm it
        1. Poke 5 little holes in the parafilm then place cap on vial
      - iii. Place vial in lyophilizing container
        1. Cushion vial with paper towels
      - iv. Turn lyophilizing machine on and set temperature to -80 °C
      - v. Add the lyophilizing container (with the cap on) and attach it to the tube
      - vi. Leave on overnight
- \*Vial now contains morpholino and salts from Danieau solution. Resuspend morpholino with 250 ul Milli Q H<sub>2</sub>O and follow “How to Make Resuspend Morpholino” protocol

### How to make 1x E3 Buffer for Standard work with Embryos

1. Find 50x E3 buffer in 4 °C fridge
2. Rinse 1x E3 bottle with dI H<sub>2</sub>O
3. Calculate the amount of 50x E3 needed to make 1000 mL of 1x E3 buffer

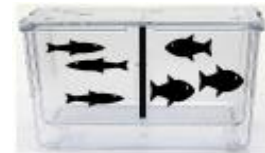
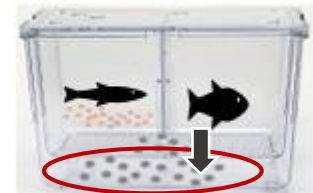
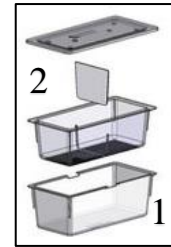
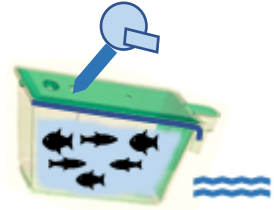
Stock	Final
(50x E3) (x)	= (1x) (1000 mL)
	$x = (1000x / \text{mL}) / 50x$
	$x = 20 \text{ mL}$
4. Add 20 mL of 50x E3 buffer to 980 mL of dI H<sub>2</sub>O to make 1L (1000 mL) of 1x E3
5. Store 1x E3 buffer in Fish Room for embryo isolation and in lab for embryo maintenance

\*Key points: E3 can be made up as a 60x stock. The 1x medium keeps under non-sterile conditions at room temperature for over a week. 60x E3 stock contains methylene blue. When zebrafish embryos are kept in 1x E3 buffer, they contain the methylene blue reagent, which suppresses fungal outbreaks in Petri dishes in the event that an egg goes bad.

## Appendix III: Embryo Isolation and Injection

### How to Set-Up Mating

1. Select a tank (Aquaneering, San Diego, CA, USA) between 2:00 pm – 4:00 pm
  - a. This allows for adequate hormone build up
  - b. Follow the order on the “Tank Mating List” in the Fish Room
    - i. Fish will need a 3-5 day rest period before another mating can be set up
2. Retrieve the tank
  - a. Remove the automatic feeder (Penn Plax Inc., Memphis, TN, USA)
    - i. Unscrew the light blue knob and remove the feeder
  - b. Turn off the water supply for that tank. Lift the tank by the front side
    - i. This lowers the water level which prevents a mess from happening when you take the tank out
  - c. Remove the tank
    - i. Lift the tank up and over the drain pipe
  - d. Place the tank on the counter near the sink
3. Set up the Mating Tank
  - a. Grab the two mating tank components (Pentair- Aquatic Habitats, Minneapolis, MN, USA)
    - i. Clear, plastic tank (#1)
    - ii. Clear, plastic tank with holes on the bottom (#2)
  - b. Insert the 2<sup>nd</sup> container into the first
    - i. This allows the fish to lay eggs that can be collected easily. The eggs will sink to the bottom, passing through the holes at the bottom of container #2, and kept isolated in container #1.
  - c. Fill the mating tank with water from the main tank set up (filtered aged tap water)
4. Transfer fish to the mating tank
  - a. Using a small black net, capture the fish and transfer them to the mating tank
  - b. Using a divider, separate the fish by sex (male or female)
  - c. Place a lid on top of the tank and put them on the shelf.
    - i. If mating more than one tank, label it with the number corresponding to the tank (#1-6)



Male Female

**Figure 1:** Distinct characteristics between Male and Female Zebrafish

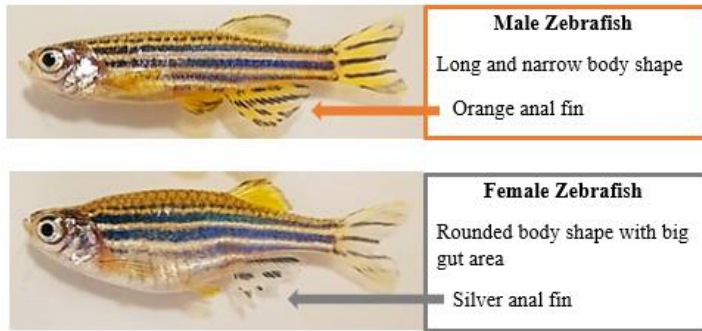


Photo credit: Michael Y.F. Yuen

5. Clean up
  - a. Rinse the net with dI H<sub>2</sub>O before returning
  - b. If the original tank is clean, return it to its designated area and turn H<sub>2</sub>O back on
  - c. If the original tank is dirty, following “How to Clean Tanks” protocol
  - d. Feed fish after separating them in mating chamber or around 8:00 pm
6. Pull the divider at 9:00 am and isolate embryos- see “How to Microinject Embryos”
  - \* If they do not lay at or around 9:30 am, check “Embryo Collection Troubleshooting Guide”

**\*\* Significant changes**

- Fish were cycled as they aged
  - A few days into the project (June 2018), 12 zebrafish were brought into our facility. Following a 2-3 weeks acclimation period, zebrafish were used for mating purposes. The 12 zebrafish were separated into two tanks: three males and three females in each tank
  - Fish over a year old were euthanized and replaced with newer fish
  - Four months into the project (October 2018), 6 zebrafish (3 males and 3 females) were brought into our facility. Following a 2-3 weeks acclimation period, zebrafish were used for mating purposes.
- There are currently only four tanks
  - Each tank contains three males and three females
- Nine months into the project, the light-dark cycle changed from “9:30 am- 11:30 pm” to “9:00am- 11:00 pm”
- Ten months into the project, the light-dark cycle changed from being dial operated to analog operated
  - This ensures better accuracy of light-dark cycle



## Embryo Collection Troubleshooting Guide

1. Pull divider at 9:00 am
2. Check for embryos at 9:30 am
  - a. If there are embryos, follow protocol
  - b. If there aren't any embryos, check again in 30 minutes
3. Check for embryos at 10:00 am
  - a. If there are embryos, follow protocol
  - b. If there aren't any embryos, check again in 30 minutes
4. Check for embryos at 10:30 am
  - a. If there are embryos, follow protocol
  - b. If there aren't any embryos, check again in 30 minutes
5. Check for embryos at 11:00 am
  - a. If there are embryos, follow protocol
  - b. If there aren't any embryos, check again in 30 minutes
6. Check for embryos at 11:30 am
  - a. If there are embryos, follow protocol
  - b. If there aren't any embryos, check again in 30 minutes
7. Check for embryos at 12:00 pm
  - a. If there are embryos, follow protocol
  - b. If there aren't any embryos, return zebrafish to original tank and rinse mating chamber with dI H<sub>2</sub>O
    - i. Record "no" under "embryos?" in the "Tank mating sheet" kept in the Fish Room
8. Check to see if food funnels are clogged
9. Check if automatic feeders are adequately filled with food
10. Check if feeders are aligned to funnels
11. Ensure tanks are clean and the "sump" is adequately filled
  - a. For instructions, please see "How to Clean Tanks" and "How to Add Water to the Fish System"

## How to Microinject Embryos

1. Pulling and loading needles- See Figure 2
  - a. Take a Borosil 1.0 MM OD x 0.5 MM ID capillary tube (FHC Inc., Bowdoin, ME, USA)
  - b. Insert the capillary tube into the grooves of the Heka Pipette Puller (Heka Instruments, Bellmore, NY, USA)
    - i. Go as far down the groove as it can go without dropping it
  - c. Tighten the first yellow bar
  - d. Lift the second yellow bar to spacer disk setting 12
    - i. Tighten the second yellow bar and remove spacer disk
  - e. Flip switch to “A,” turn power on, and hit START
    - i. Red coil appears when heating then cools back down
  - f. Flip switch to “off,” loosen the first yellow bar and lift it to spacer disk setting 5
    - i. Tighten the yellow bar and remove spacer disk
  - g. Flip switch to “B” and hit START
    - i. The coil will turn red when heating, then cool down
  - h. Loosen the first yellow bar
    - i. Remove top pipette
      1. Dispose of this pipette in “sharps” box
  - i. Loosen the bottom yellow bar while holding onto the pipette
    - i. Carefully remove the pipette
      1. THIS NEEDLE WILL BE USED FOR MICROINJECTING EMBRYOS
  - j. Place newly made pipette on clay (let it hang from the shelf) until ready to load
    - i. When you are ready, use a P2 pipette to pipette 1.5 ul of what you will inject into the embryos
      1. For Morpholino, follow Step #2
      2. For Sham injection (phenol red), follow Step #3

## 2. Preparing Morpholino injections

- a. Go to freezer (-20°C) and find “Scyba MO” box (3<sup>rd</sup> shelf)
- b. Pick a 5 ul aliquot of Morpholino (Gene Tools LLC., Philomath, OR, USA) and put box back in the freezer
- c. Add 5 ul of phenol red injection buffer (Sigma-Aldrich, St. Louis, MO, USA) to the morpholino aliquot to make the final volume 10 ul
- d. Quick spin in centrifuge (SciLogex LLC, Rocky Hill, CT, USA)
- e. Load the pipette that was made in the pipette puller with 1.5 ul of Morpholino
  - i. Let the Morpholino reach the tip of the needle
- f. Cover Eppendorf cap with parafilm and store the MO solution in the 4°C fridge
  - i. This is to prevent evaporation

\*For information on morpholino, see “How to Resuspend Morpholino”

3. Preparing Sham injections
  - a. Add 5 ul of 1x Danieau solution to 5 ul of phenol red (1:1 ratio)
 

\*This is a troubleshooting attempt to prevent high levels of embryo mortality

    - i. Originally, you would only use phenol red as the injection buffer. This resulted in high levels of embryo mortality. Since morpholino is resuspended in 1x Danieau solution, we decided to mix equal parts of phenol red with equal parts of 1x Danieau solution.
    - ii. For instructions, see “How to make 1x Danieau solution”
4. Microinjection Set-up- See Figures 2 and 3
  - a. Turn on Fiber-Lite High Intensity Illuminator Series 180 (Dolan-Jenner Industries, Boxborough, MA, USA)
    - i. Keep intensity at “4”
    - ii. This is the microscope light
  - b. Turn on Leitz AC volts light source (E. Leitz Inc., New York, NY, USA)
  - c. Turn on Picospritzer III (Parker Hannifin Corporation, Pine Brook, NJ, USA)
    - i. Turn switch to “MSEC”
      1. This is milliseconds
      - ii. This controls the duration at which microinjection occurs
  - d. Turn on gas tank (Airgas Inc., Radnor, PA, USA)
    - i. This allows air to pass through the pipette for microinjection
  - e. Carefully insert the pipette from Step 1 into the micromanipulator (Narishige International Inc., Amityville, NY, USA)
    - i. Make sure it is loaded- either Step 2 or 3
5. Mineral Oil Test
  - a. Once the pipette is inserted into the micromanipulator, run the very tip of the needle against a small pumice stone
 

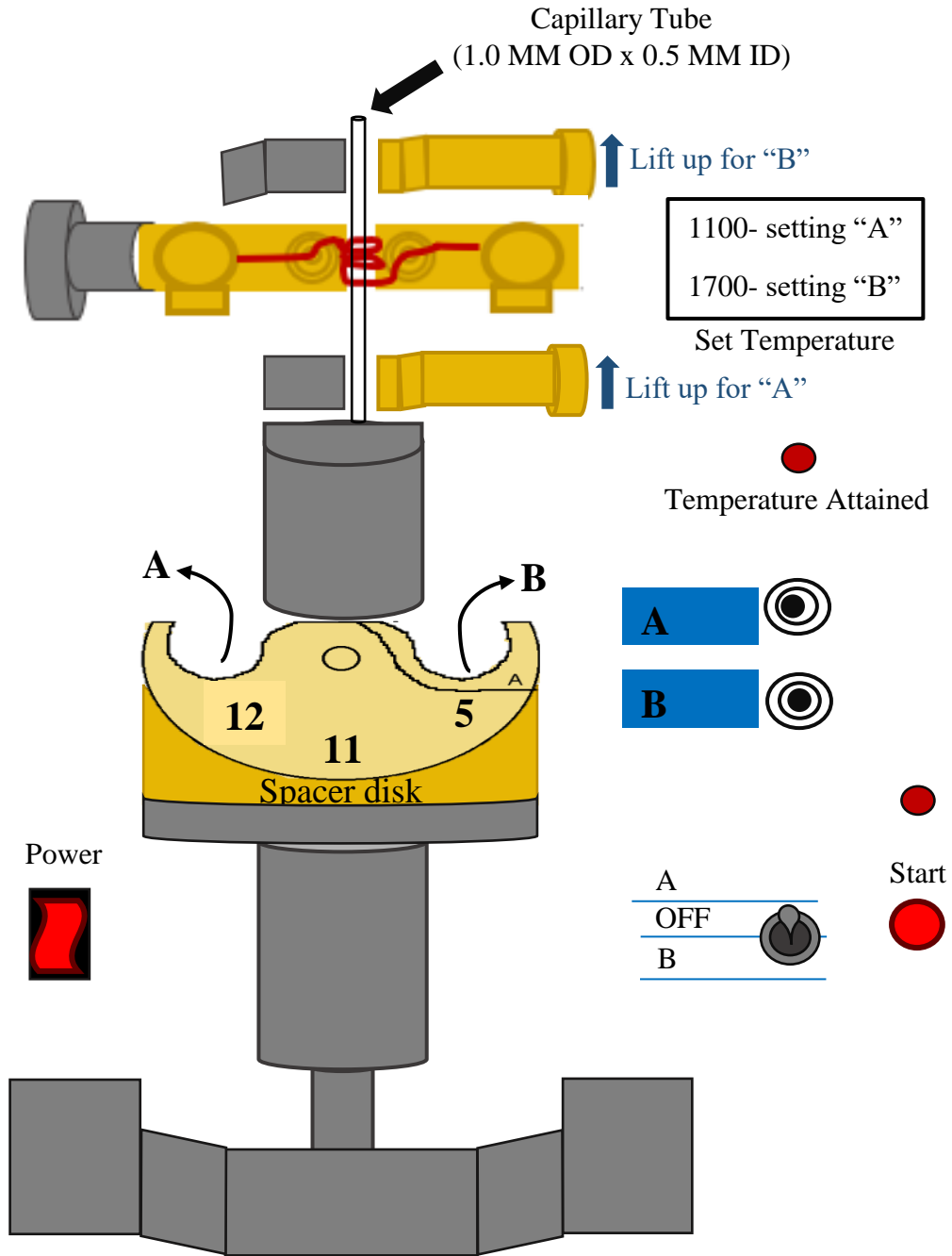
\*This is a change that results in a more controlled break of the tip. Before, you would use a pair of forceps to make a small break at the tip of the needle. By using a pumice stone, you simply run it against the stone softly.
  - b. Use a P2 pipette tip to “scoop” a large drop of mineral oil
  - c. Add the drop of mineral oil onto the middle of the black circle in the mineral oil slide.
    - i. The mineral oil slide is kept in a small black case in the drawer labelled “Microinjection supplies”
  - d. Bring the micromanipulator close to the microscope so that the needle is in the mineral oil
    - i. Adjust as necessary
  - e. Step on the foot pedal (located under the bench). Air will pump through the needle and onto the mineral oil slide

- i. Depending on how big your bubble is, decide if the pipette is fine or needs to be broken a bit more
    - ii. Worst case scenario, your needle breaks more than you anticipated and you will need to start the process over
    - iii. Ideally, you want the bubble to be between 2 spaces in width (2 spaces in width= 1 nl)
      - 0.1 mm= 0.5 nl, so 0.2 mm= 1 nl
  - f. Once you have your ideal needle, bring the embryos and store mineral oil slide in drawer labelled “Microinjection supplies”
    - i. Clean off the mineral oil that is on the slide with a kimwipe (Kimtech Inc., Vaughan, Ontario, CA) and put it back in its case
6. Isolating Embryos
- a. Pull the mating tank from the shelf and place it on the counter
  - b. Fill a separate mating chamber (just the container without holes at the bottom) with filtered aged tap water. Place this mating chamber on the counter near the sink
  - c. Lift the container with holes in the bottom and transfer it to a new mating chamber
    - i. This allows you to continue collecting embryos in case the zebrafish are not done laying
  - d. Grab the fine strainer and hold it over the sink
  - e. Pour water from the mating tank through the strainer and into the sink
    - i. Since you are using a fine strainer, the embryos are just big enough to not flow through.
    - ii. Since some debris will stick around, use a squirt bottle to remove feces, debris, etc. from the strainer
      - 1. The squirt bottle should always be filled with 1x E3 buffer and kept in the fish room
        - a. For instructions, please see “How to Make 1x E3 Buffer”
  - f. Once there are only embryos in the strainer, grab a 100 x 15 mm petri dish (VWR International, Radnor, PA, USA) and add some 1x E3 buffer to the dish
  - g. Flip the strainer over the dish. Using the squirt bottle to push the embryos from the strainer onto the dish
    - i. The force of the squirt is enough to push the embryos off the strainer and into the petri dish
  - h. Cover the petri dish with a lid and label (AC, date, time, tank #)
  - i. Record “Yes” under “embryos?” for corresponding tank number on the sheet
  - j. Place the mating chamber back on the shelf
    - i. Zebrafish typically lay every 20-30 minutes for a set period of time
      - 1. If not, please see “Embryo Isolation Troubleshooting Guide”

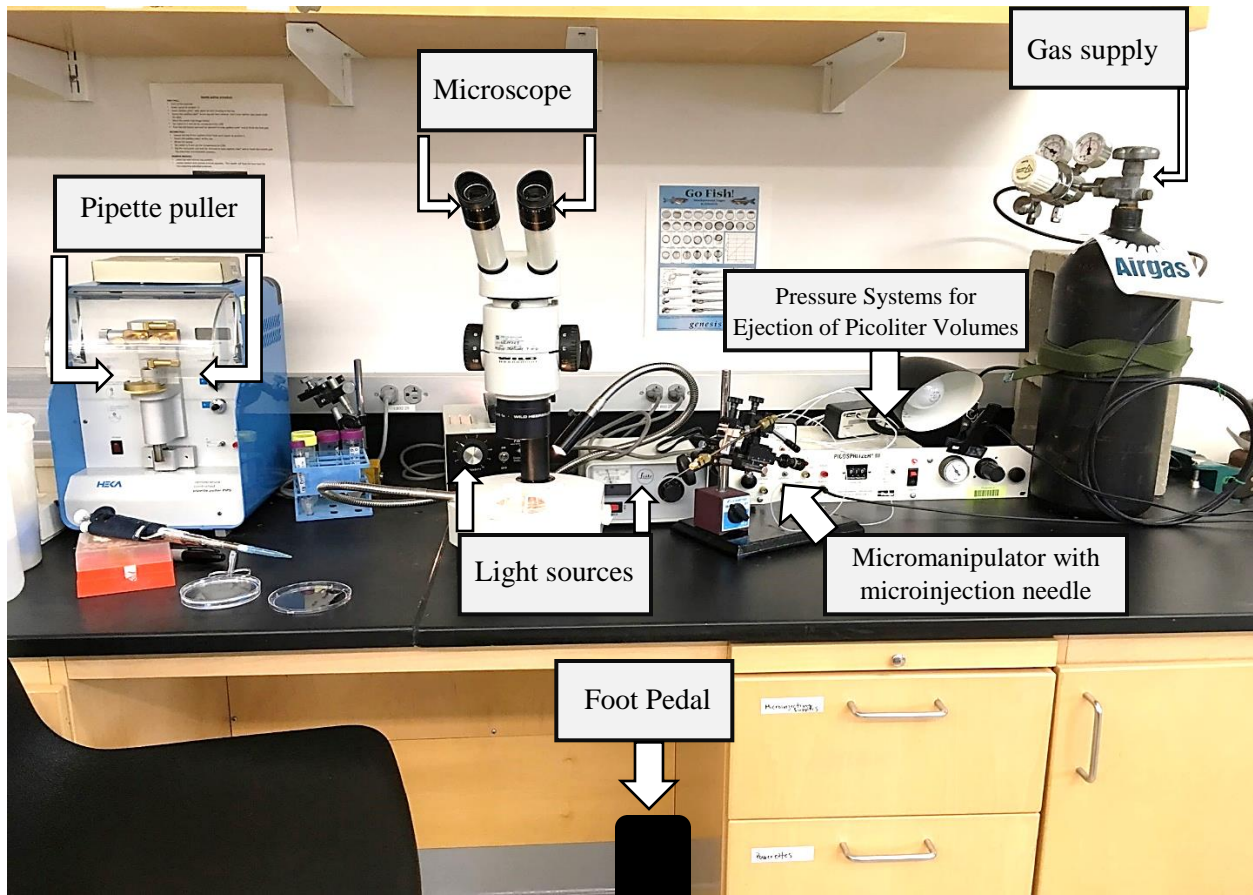
- k. Microinject embryos 30 minutes after laying to ensure proper injection
7. Injecting Embryos- See Figure 4
- a. Grab a P1000 and a P1000 pipette tip
    - i. Cut the end of the pipette tip to make the opening larger
  - b. In a drawer labelled “Microinjection supplies,” grab a petri dish and put a normal slide inside
  - c. Using the P1000, pipette embryos onto the petri dish along the edge of the slide
    - i. Tilt the dish to the side so water flows through the slide, leaving embryos aligned along the straight edge
      - 1. Remove water as necessary
  - d. Once all the embryos are aligned, place the petri dish under the microscope and bring the micromanipulator (with the loaded pipette) to the microscope and dish
  - e. Use the microscope to adjust the needle so it can puncture the embryo at the right spot in the yolk- see Figure 4
    - i. Careful to adjust the needle as much as possible before puncturing the embryo. If you adjust the needle while it is in the embryo, you will cause significant damage. Align it to where the needle will puncture into the yolk, not just the chorion itself
  - f. Hit the foot pedal and air will pump through the pipette to inject the embryo
    - i. To see if the embryo is injected, look in the microscope. Embryos should have a spot of pink-red hue inside
  - g. Go down the line of embryos by moving the dish to and from the pipette and inject all the embryos
  - h. Once all the embryos are injected, use forceps to lift the slide off the dish
    - i. Begin by lifting the corner of the slide. Once the slide is upright, hold it steady with your fingers and lift up
    - ii. Be careful not to damage any embryos in the process. To avoid this, do not drop the slide after it is in an upright position.
    - iii. If any embryos are stuck to the slide, use a squirt bottle with 1x E3 buffer to push them back onto the dish.
  - i. Fill the petri dish with 1x E3 buffer and put a lid on it. Label the lid (AC, date, time, # of embryos, type of injection, incubation period)
  - j. Keep the petri dish in the 28°C incubator (Benchmark Scientific Inc., Sayreville, NJ, USA)
  - k. Repeat this process for every batch of embryos you get. Be sure to isolate and inject embryos 20-30 minutes after they have been laid.
    - i. This is to ensure embryos are injected at the 1-cell stage
8. Turn it off
- a. Turn off Fiber-Lite High Intensity Illuminator and Leitz AC volts light sources
  - b. Flip switch to “MIN” in PICOSPRITZER III

- c. Turn off Airgas
  - d. Discard needle into “sharps” box
  - e. Hit the foot pedal. The gas should release for 1 minute
    - i. Gas valves should go down to zero
  - f. Turn off PICOSPRITZER III and flip switch back to “MSEC”
9. Make sure zebrafish are put back into the original tank
- a. Turn the water for the original tank off. Place the tank on the counter. Remove the lid.
  - b. Lift the mating tank (with holes on the bottom) and align it to the original tank
  - c. Tilt the mating tank so that the fish can go back in the tank
  - d. Place the lid back on the original tank and return it to its designated area
  - e. Turn the water supply back on, place the food funnel in the designated area and align the automatic fish feeder above the funnel
    - i. Ensure that the feeder is full of food and aligned properly
10. Rinse and air dry
- a. Rinse mating tanks (both containers) and strainer with dI H<sub>2</sub>O. Allow them to air dry on rack
11. Periodically check on embryos
- a. Ensure they have fresh 1x E3 buffer
  - b. Remove any unviable embryos
  - c. Remove any hyphae growing and replace buffer with fresh 1x E3 buffer.

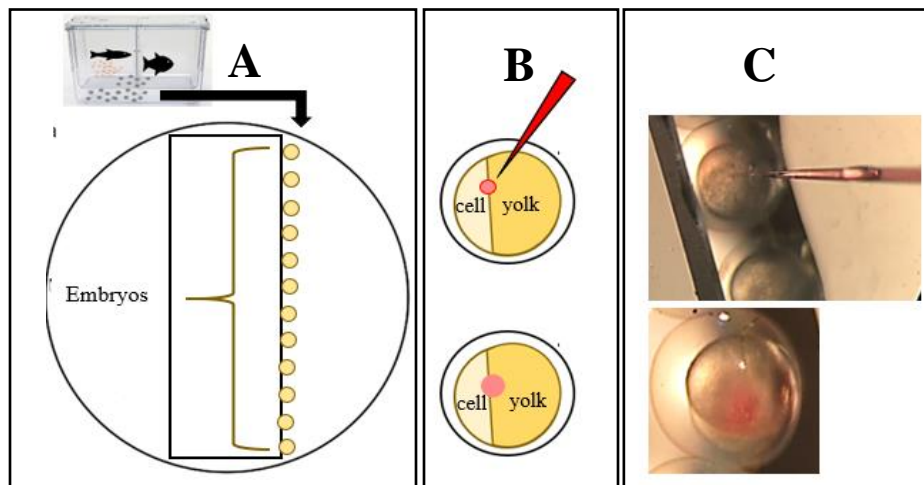
**Figure 2: Pulling Pipettes**



**Figure 3: Microinjection Station**



**Figure 4: Microinjecting Embryos**



**Figure 4. Microinjecting Embryos.** (A) Align embryos along the edge of a microscope slide inside a petri dish. (B) Adjust the microinjection needle to where it can puncture the embryo at the right spot inside the yolk. (C) Microinject embryos at the one-cell stage (30 minutes post-fertilization) inside the yolk by the cell.



## Appendix IV. Molecular Techniques

### RNase-Free Zone

1. Obtain a 1500 mL flask and dilute 10 M NaOH into 0.2 M concentration
  - a.  $M_1V_1 = M_2V_2$ 
    - i.  $(1400 \text{ mL})(0.2 \text{ M}) = (10 \text{ M})(?)$
    - ii.  $280 \text{ M/mL} = (10 \text{ M})(?) \rightarrow (280/10 \text{ M})$
    - iii.  $V_2 = 28 \text{ mL}$
2. Use a pipette gun and 10 mL “baked for RNA work” pipettes to pipette 28 mL of 10 M NaOH into the empty flask.
  - a. Discard pipette in 2% bacdown 5/24 pipette jar
3. Add 1372 mL deionized H<sub>2</sub>O into the flask and swirl
4. Pour solution into a 1500 mL beaker
5. Take pipettes apart
  - a. Let pipette barrels soak in 0.2 M NaOH for ~1 hour
6. Obtain 1000 mL of 95% EtOH
7. After ~1 hour of pipette barrels soaking in 0.2 M NaOH, pour 0.2 M NaOH solution into the original 1500 mL flask. Label (0.2 M NaOH + dI H<sub>2</sub>O AC 6/14)
8. Rinse beaker and pipette barrels with Milli Q H<sub>2</sub>O
9. Transfer 1000 mL EtOH from the flask into the beaker
  - a. Let pipette barrels soak in 95% EtOH for ~30 minutes
10. After soaking in 95% EtOH for ~30 minutes, rinse pipette barrels with Milli Q H<sub>2</sub>O and leave to air dry in RNase-free zone
11. Transfer 95% EtOH from beaker into the original flask and label (95% EtOH AC 6/14)
12. Rinse beaker with Milli Q H<sub>2</sub>O and leave to air dry
13. Wipe down RNase-free area thoroughly
  - a. Wipe pipette parts, boxes of pipette tips, bottles with solutions, tubes with solutions, gel box, gel tray, gel comb, lab bench, etc... with 0.2 M NaOH
  - b. Following 0.2 M NaOH treatment, wipe items and bench space with 95% EtOH and leave them to air dry

\*Key Points: NaOH degrades RNases, 95% EtOH gets rid of base and sterilizes everything

## RNA Isolation

RNA isolation prepared using the Direct-zol RNA Mini Prep kit (Zymo Research, Irvine, CA, USA) in an RNase-free station with aerosol-resistant pipette tips

\*\* All centrifugation steps are done at 10,000-16,000 x g for 30 seconds

1. Add ethanol to buffers (this step only needs to be done once)
  - a. Add 10 mL of 95-100% EtOH to “Direct-zol RNA Pre-Wash
  - b. Add 48 mL of 100% EtOH to “Direct-zol RNA Wash Buffer”
    - i. Use the pipette gun and a 10 mL baked pipette to add 100% ethanol
      1. Discard pipette in 2% bacdown pipette jar
2. Check embryos under microscope
  - a. Discard any ruptured, unfertilized, or unviable embryos. Ensure proper incubation period is achieved before proceeding
3. Lyse and homogenize embryos in appropriate volume of TRI Reagent
  - a. For cells  $\leq 10^6$ , add 250 ul TRI Reagent
    - i. 50 embryos are needed per incubation period within each time point (Peterson and Freeman, 2009)
      1. If there are less than 50, preserve them using dry ice and ethanol.  
For instructions, see “RNA Isolation Troubleshooting Guide”
    - ii. Homogenize embryos with baked RNase-free homogenizers
4. Centrifuge sample to remove particulate debris and transfer the supernatant into a new, RNase-free Eppendorf tube
5. Add an equal volume of 95-100% ethanol to the supernatant (250 ul) and mix thoroughly
6. Transfer solution into a Zymo-Spin IIC Column in a collection tube and centrifuge
  - a. Discard flow through
7. DNase I Treatment
  - a. Add 400 ul of RNA Wash Buffer to the column and centrifuge
    - i. Discard flow through
  - b. In RNase-free Eppendorf tube, add 5 ul of DNase I and 75 ul of DNA Digestion Buffer. Mix thoroughly
    - i. Add the mix directly to the column matrix. Careful not to puncture column
  - c. Incubate at room temperature for 15 minutes
8. Following the incubation period, add 400 ul of Direct-zol RNA Pre-Wash to the column and centrifuge
  - a. Discard flow through
9. REPEAT STEP #8
10. Add 700 ul of RNA Wash Buffer to the column and centrifuge for 2 minutes to ensure complete removal of the wash buffer
11. Transfer column into an RNase-free tube. Label (AC RNA #, date, type of injection)
12. To elute RNA, add 100 ul DNase/RNase-free H<sub>2</sub>O to the column matrix and centrifuge

13. Record RNA concentration and purity using the NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA)
  - a. For list of RNA concentrations, please see “How to use the NanoDrop”
14. Store RNA in -80°C freezer, Room #312
  - a. Stored in “Section 3, Row 2” in the box labelled “Ariana Embryo Work”
15. Once you have RNA isolated from every time point and injection, run an RNA gel
  - a. By loading RNA into a gel, we can visualize and compare the size of the bands to the concentration values from the Nanodrop. This is also used as a quality control method to determine whether RNA is degraded or not.
    - i. For instructions, please see “Gel Electrophoresis”

### **RNA Isolation Troubleshooting Guide**

1. Based off the literature, 50 zebrafish embryos are needed for RNA isolation (Peterson and Freeman, 2009)
2. If there are not enough viable embryos to fulfill this requirement, freeze embryos with dry ice and store in -80°C freezer
  - a. After several attempts, it can be concluded that the appropriate amount of ethanol should be added in before flash freezing embryos with dry ice.
3. **DO NOT FLASH-FREEZE EMBRYOS IN TRIZOL.**
  - a. This resulted in low RNA concentration

250 ul Trizol or Ethanol/ 50 embryos= 5 ul of Trizol or Ethanol per embryo

### **How to Bake Homogenizers**

1. Select three glass homogenizers
  - a. Assign one homogenizer for each type of injection: non-injected, sham injected, and morpholino injected
2. Rinse with soap and dI H<sub>2</sub>O after each use
3. Allow homogenizers to air dry
4. Once dry, wrap homogenizers and respective pestles in aluminum foil
  - a. To denote “morpholino-injected” pestle and homogenizer, color the end of the aluminum foil with a red sharpie
5. Place the pestles and homogenizers (wrapped in aluminum) in the oven (Blue M Electric, Blue Island, IL, USA) located in the stockroom
6. Set time for 6.5 hours to allow for proper elimination of DNA nucleases
  - a. It takes ~1 hour to reach the temperature necessary to get rid of nucleases. The oven needs 4 hours to bake. This method is used for dry, hot sterilization
7. Handle with gloves and keep in an RNase-free station

### How to use the Nanodrop

1. Take an RNase-free pipette and P2 aerosol-resistant pipette tips. Keep RNA on ice.  
Wear gloves at all times
  - a. Go to room #330A to use the NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA)
2. Go to the computer, click “start” and select “NanoDrop 2000”
3. You will be taken to the NanoDrop homepage. For RNA analysis, click “Nucleic Acid”
4. On the right-hand side of the screen is “type.” Click it and find “RNA.” Choose this option
5. For “sample ID,” type “Milli Q” and load the blank solution
  - a. Pipette 1 ul RNase-free Milli Q on the nanodrop platform
  - b. Lower the arm pedestal
6. Click on “blank”
  - a. Now that the NanoDrop is cleaned and blanked, lift the arm pedestal and wipe the upper (arm) and lower (platform) pedestal with a kimwipe
  - b. Be sure to clean the pedestal with a clean kimwipe after each sample is measured, even if it is just Milli Q.
7. For safe measure, load 1 ul of Milli Q H<sub>2</sub>O and measure
8. Clean pedestal and load 1 ul of RNA sample
  - a. Type “RNA #, date, type of injection” for “sample ID”
  - b. Pull down the arm and click on the “measure” button
9. Lift the arm up, wipe the pedestal, and load 1 ul of Milli Q H<sub>2</sub>O
  - a. Pull the arm down and click on the “measure” button
10. Clean the pedestal and repeat steps #7-9 until all RNA samples are measured. If there are no more RNA samples left to measure, end the process by loading 1 ul of Milli Q H<sub>2</sub>O and wiping the pedestal
11. Record and save data
  - a. To save the data, go to “Report” and highlight the samples you want to save
  - b. Click on the “export” button
  - c. Where it says “My Documents,” click the drop down arrow and select “Nanodrop data”
  - d. Create a new folder (Ariana- Wall RNA)
  - e. For “filename,” type “RNA #, Date, Type of injection”
  - f. For “save as,” select “Spectra New Workbook (\*.twbk) and click “save”
  - g. Close out of the program and store RNA in the -80°C freezer

\*Key points: The NanoDrop is used to measure RNA concentration and purity. Ideally, an RNA A260/280 ratio around 1.8-2.0 indicates pure RNA. By having RNA concentrations (ng/ul), we can calculate how much RNA to use for cDNA synthesis. This ensures an equal amount of RNA is pipetted into each tube for cDNA synthesis.

### RNA Batch #1

Date	Type of Injection	Time Point	RNA #	Nucleic acid concentration	A260/280
7/5	Sham	24 hr	1	155.1 ng/ul	2.04
7/19	Non	36 hr	2	274.7 ng/ul	2.02
7/23	Morpholino	24 hr	4	136.3 ng/ul	2.06
7/23	Morpholino	36 hr	5	151.6 ng/ul	2.05
7/24	Sham	36 hr	6	90.2 ng/ul	2.10
7/25	Non	24 hr	3	188.8 ng/ul	2.05
7/31	Sham	36 hr	6	153.7 ng/ul	2.02
10/1	Non	48 hr	7	69.0 ng/ul	2.06
10/1	Sham	48 hr	8	337.6 ng/ul	2.00
10/1	Morpholino	48 hr	9	9.8 ng/ul	2.09
10/16	Non	48 hr	7	145.6 ng/ul	2.04
10/16	Morpholino	48 hr	9	145.8 ng/ul	2.01

\*Added 48-hour time point in October 2018

**RNA Batch #2**

Date	Type of Injection	Time Point	RNA #	Nucleic acid concentration	A260/280
1/14	Non	24 hr	3	150.5 ng/ul	2.03
1/14	Morpholino	24 hr	4	109.0 ng/ul	2.05
1/14	Sham	48 hr	8	142.8 ng/ul	1.99
1/15	Non	36 hr	2	245.2 ng/ul	2.03
1/15	Morpholino	36 hr	5	162.3 ng/ul	2.01
1/15	Non	48 hr	7a	278.7 ng/ul	1.99
1/15	Non	48 hr	7b	205.7 ng/ul	1.99
1/15	Non	48 hr	7c	212.8 ng/ul	1.98
1/15	Non	48 hr	7d	295.1 ng/ul	1.94
1/13	Sham	24 hr	1	163.4 ng/ul	1.99
2/11	Sham	36 hr	6	236.2 ng/ul	2.01
2/11	Morpholino	48 hr	9	193.7 ng/ul	2.00

**RNA Batch #3**

Date	Type of Injection	Time Point	RNA #	Nucleic acid concentration	A260/280
2/11	Non	36 hr	2	274.0 ng/ul	2.00
3/30	Non	24 hr	3	284.3 ng/ul	2.00
3/30	Sham	24 hr	1	270.0 ng/ul	2.03
3/30	Morpholino	24 hr	4	205.7 ng/ul	2.05

## cDNA Synthesis

\*Synthesize cDNA in an RNase-free environment. Use aerosol resistant barrier pipette tips

1. Calculate the amount of RNA (ul) needed for the cDNA synthesis reaction
  - a. Calculations
    - Volume for cDNA synthesis (x ul) \* [sample] = 1500 ng (1.5 ug)
    - i. Plug in RNA concentration value in [sample]. Divide 1500 ng by RNA concentration to solve for “x” (volume needed for cDNA synthesis in ul)
  - b. From there, calculate the amount of RNase-free H<sub>2</sub>O needed to bring the reaction total up to 12.5 ul.
 

12.5 ul reaction – calculated RNA volume (ul) = x ul RNase-free H<sub>2</sub>O
  - c. Repeat calculations for each time point within each injection type

Component	Final Concentration	Volume- 1x MM	9.5x MM
Oligo dT (10 uM)	0.5 uM	1 ul	9.5 ul
dNTP (10 mM)	500 uM	1 ul	9.5 ul
5x RT Buffer	1x	4 ul	38 ul
RNasin (40 U/ul)	20 U/rxn	0.5 ul	4.75 ul
Easy Script RTase (200 U/ul)	200 U/rxn	1 ul	9.5 ul
RNase-free H <sub>2</sub> O	---	x ul	x ul
RNA	0.5-5 ug/rxn	x ul	x ul
<b>Total: 20 ul cDNA mix</b>			

2. Retrieve RNA from -80°C freezer
3. Thaw RNA and cDNA synthesis reagents (Lamda Biotech Corporation, Ballwin, MO, USA) on ice
  - a. Oligo dT
  - b. dNTP
  - c. 5x RT Buffer
  - d. RNasin
  - e. Easy Script RTase
  - f. RNase-free H<sub>2</sub>O
4. Mix each solution by vortexing for 10 seconds
5. Briefly centrifuge to collect residual liquid from the sides of the tubes
6. Prepare reaction mixture on ice in PCR tubes
7. Prepare a 9.5 master mix (MM) of solutions (excluding RNA and RNase-free H<sub>2</sub>O)

- a. This step has been revised. Adapt master mix needs based off how many samples there are. For the total number of cDNA reactions you plan to run, add an extra 0.5 “reaction.” This ensures there will be enough reagents in the master mix for every reaction. This is because there is always a window of error that can occur when using pipettes.
8. Pipette 7.5 ul of 9.5 MM into each PCR tube
9. Label PCR tubes “3, 1, 4, 2, 6, 5, 7, 8, 9” to dictate which RNA sample corresponds with which PCR tube
10. Pipette RNA and RNase-free water to the corresponding tubes (match calculated volumes to RNA number on PCR tubes)

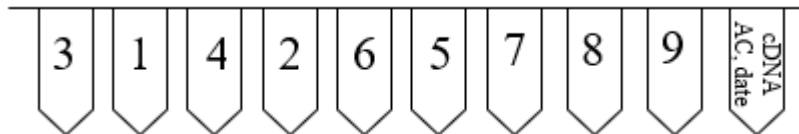
<b>Batch #1</b>		
<b>RNA # (Injection)</b>	<b>Volume (ul)</b>	<b>RNase-free H<sub>2</sub>O (ul)</b>
RNA 3 (Non 24)	7.944 ul	4.556 ul
RNA 1 (Sham 24)	9.671 ul	2.829 ul
RNA 4 (MO 24)	11.005 ul	1.495 ul
RNA 2 (Non 36)	5.460 ul	7.04 ul
RNA 6 (Sham 36)	9.759 ul	2.741 ul
RNA 5 (MO 36)	9.894 ul	2.606 ul
RNA 7 (Non 48)	10.30 ul	2.2 ul
RNA 8 (Sham 48)	4.44 ul	8.06 ul
RNA 9 (MO 48)	10.28 ul	2.22l



<b>Batch #2</b>		
<b>RNA # (Injection)</b>	<b>Volume (ul)</b>	<b>RNase-free H<sub>2</sub>O (ul)</b>
RNA 3 (Non 24)	9.966 ul	2.534 ul
RNA 1 (Sham 24)	9.179 ul	3.321 ul
RNA 4 (MO 24)	13.76 ul	0 ul
RNA 2 (Non 36)	6.117 ul	6.383 ul
RNA 6 (Sham 36)	6.35 ul	6.15 ul
RNA 5 (MO 36)	9.24 ul	3.26 ul
RNA 7a (Non 48)	5.382 ul	7.118 ul
RNA 7b (Non 48)	7.29 ul	5.21 ul
RNA 7c (Non 48)	7.048 ul	5.452 ul
RNA 7d (Non 48)	5.083 ul	7.417 ul
RNA 8 (Sham 48)	10.504 ul	1.996 ul
RNA 9 (MO 48)	7.744 ul	4.756 ul

<b>Batch #3</b>		
<b>RNA # (Injection)</b>	<b>Volume (ul)</b>	<b>RNase-free H<sub>2</sub>O</b>
RNA 3 (Non 24)	5.276 ul	7.224 ul
RNA 1 (Sham 24)	5.555 ul	6.945 ul
RNA 4 (MO 24)	7.292 ul	5.208 ul
RNA 2 (Non 36)	5.474 ul	7.026 ul

11. Set up the Thermocycler (Eppendorf AG, Hamburg, DEU)
    - a. User: Nancy
      - i. Hit “enter” when prompted for password
    - b. Find folder labelled “Nancy”
      - i. Add a new folder
        1. Set up cDNA reaction- labelled “cDNA AC”
          - a. 42°C for 60 minutes
            - i. This will be the incubation period
          - b. 85°C for 5 minutes
            - i. This will be to stop the reaction
          - c. Chill at -20°C
            - i. This is to preserve reactions until it can be stored in the -20°C freezer
12. Run cDNA synthesis
  - a. Find folder labelled “Nancy”
    - i. Select “cDNA AC”
  - b. Check thermocycler settings
    - i. Incubate at 42°C for 60 minutes
    - ii. Heat reaction at 85°C for 5 minutes
    - iii. Chill on ice
  - c. Hit “start”
13. Store until ready for use
  - a. Store in freezer at -20°C until ready to run qPCR
  - b. cDNA reactions stored in “Ariana box of primers”



### Primer Design

Set #1: We read through the literature and identified possible genes in zebrafish that are affected by, or downstream of, either CXCL14 or CXCL12.

<b>Primer Design Set #1</b>		
	<b>Gene</b>	<b>Description</b>
<b>Control</b>	B-actin 1: Beta-actin 1	reference gene control
<b>Control</b>	GAPDH: Glyceraldehyde 3-phosphate dehydrogenase	reference gene control
<b>DT of CXCL14</b>	NF-κB (Nuclear factor kappa B p105)	CXCL14 up-regulates activation of NF-κB
	SCX-a (Scleraxis bHLH transcription factor a)	CXCL14 overexpression in chick embryo fibroblasts upregulates connective tissue gene expression
	Pmp-22b (Peripheral Myelin Protein 22b)	CXCL14 modulates expression myelin genes in Schwann cells, alters cell proliferation
	Mpz (Myelin Protein Zero)	CXCL14 modulates expression myelin genes in Schwann cells, alters cell proliferation
	Mbp-a (Myelin Basic Protein a)	CXCL14 modulates expression myelin genes in Schwann cells, alters cell proliferation
<b>DT of CXCL12</b>	OSR1 (Odd-skipped related 1)	CXCL12 overexpression in chick fibroblasts upregulates connective tissue gene expression
	OSR2 (Odd-skipped related 2)	CXCL12 overexpression in chick fibroblasts upregulates connective tissue gene expression
	COL17A1b (Collagen Type 17 alpha 1b)	Expressed in lateral line, neuromasts. Knockdown, reduced neuromasts in lateral line

## Primer Design

Set #2: Based off inconclusive data from “Primer Design Set #1,” we decided to modify our research question. We read through the literature and identified possible genes of interest in zebrafish that emerge during lateral line development.

<b>Primer Design Set #2</b>		
	<b>Gene</b>	<b>Description</b>
<b>Control</b>	B-actin 1: Beta-actin 1	reference gene control
<b>Control</b>	GAPDH: Glyceraldehyde 3-phosphate dehydrogenase	reference gene control
<b>Ligands</b>	CXCL14 (Chemokine ligand 14 a)	Predicted to have chemokine activity, involvement in cell chemotaxis and involvement with immune responses
	CXCL12/SDF1 (chemokine ligand 12 a; stromal cell-derived factor 1)	Exhibits chemoattractant activity; involved in animal organ development, nervous system development, and regulation of cell motility
<b>Receptors</b>	CXCR4 (chemokine receptor 4b)	Exhibits G protein-coupled chemoattractant receptor activity; involved in gamete generation, nervous system development, and regulation of chemotaxis
	CXCR7B/ackr3b (chemokine receptor 7b; atypical chemokine receptor 3b)	Exhibits chemokine binding activity; involved in blood vessel morphogenesis, chemotaxis, and nervous system development
<b>Genes expressed in neuromasts and primordium</b>	Snail-1b (snail family zinc finger 1b)	Predicted to have DNA binding activity and transcription factor activity; involved in ameboidal-type cell migration, cardiac muscle progenitor cell migration involved in heart field formation, and negative regulation of cell-cell adhesion
	Cldn-2 (Claudin 2)	Predicted to have structural molecule activity; predicted to localize to the bicellular tight junction, integral component of membrane, and plasma membrane
	Epcam (epithelial cell adhesion molecule)	Involved in cell-cell adhesion, epiboly involved in gastrulation with mouth forming second, and liver development

1. Go to the NCBI website (National Center for Biotechnology Information, Rockville Pike, MD, USA) → [ncbi.nlm.nih.gov](http://ncbi.nlm.nih.gov)

a. Click on “All databases” and scroll down to “taxonomy”

i. Type in “zebrafish” in the search box, then hit “search”

b. You will be taken to:

[Danio rerio](#) ← click here

(zebrafish), species, bony fishes

[Nucleotide Protein](#)

c. You will be redirected to the “taxonomy browser → Danio rerio” page

i. To the far right is “Entrez records”

1. Find “gene [100,231](#)” under database name

2. Click on [100,231](#)

Entrez Records	
Database Name	Direct Links
<a href="#">Gene</a>	<a href="#">100,231</a>

d. You will be redirected to “gene txid 7955 [organism: no exp]”

i. This tells us it is a zebrafish

e. Type in gene txid 7955 [organism: no exp] AND type in gene of interest here. Hit search

i. i.e. gene txid 7955 [organism: no exp] AND mpz (Primer design set #1)

ii. i.e. gene txid 7955 [organism: no exp] AND CXCL14 (Primer design set #2)

f. You will be taken to the gene result page

i. If there is more than 1 result (i.e. Scx-a vs scx-b or CXCL14-a vs CXCL14-b), read through the literature... notably the “materials and methods” section to see what they picked and why.

g. Note “gene ID” number

h. Scroll down to “genomic regions, transcripts, and products”

i. Hit [FASTA](#)

i. You will be taken to the FASTA sequence

i. Note NCBI reference sequence

1. i.e. NC\_007113.7 for mpz (Primer design set #1)

2. i.e. NM\_131627 for CXCL14a (Primer design set #2)

ii. on the top right corner is

“change region shown”

1. Click on “selected region”

2. subtract 500 bp from the “from” value and add it to the “to” value.

3. Hit “update view”

Change region shown °Whole sequence *Selected region From: _____ to: _____ Update view
--

a. This is so you can be a little upstream and downstream

iii. On the right is “Analyze this sequence”

Analyze this sequence <a href="#">Run BLAST</a> <a href="#">Pick Primers</a>
--

1. Click "[Pick primers](#)"
- j. You will be redirected to the "NCBI/Primer-BLAST" homepage
  - i. Under "[PCR template](#)," is an accession number
    1. Record it
      - a. i.e. For mpz: NC\_007113.7 (Primer design set #1)
      - b. i.e. For CXCL14-a: NM\_131627 (Primer design set #2)
    - ii. Under "[Primer parameters](#)," change PCR product size "Max" from "1000" to "500"
      1. Keep "Min" at "70"
      2. Under "[Primer pair specificity checking parameters](#)," ensure that "Database" is "refseq mRNA" and "organism" is "7955" for Zebrafish
        - a. Change "Max target size" to "1000"
      3. Hit "get primers"
  - k. After lots of waiting... you will be taken to "NCBI/Primer-BLAST: results"
    - i. You will be given "[Detailed primer reports](#)"
      1. Pick the BEST primer choice.
      2. Criteria:
        - a. Length of ~18-24 bases
        - b. 40-60% GC content
        - c. Start and end with 1-2 G/C pairs
        - d. Melting temperature (T<sub>m</sub>) of 50-60°C
        - e. Primer pairs should have a T<sub>m</sub> within 5°C of each other
        - f. Primer pairs should not have complementary regions
          - i. Pick the primers with the least amount of self-complementary values
      3. Record "forward primer" and "reverse primer" sequences as well as "fragment size"
2. Repeat the process for all genes of interest so there is a primer set designed for each gene

<b>Primer Design Set #1: General Information</b>				
	<b>Gene</b>	<b>Gene ID</b>	<b>Accession #</b>	<b>Annealing temp for PCR amplification</b>
<b>control</b>	B-actin 1	57934	AF057040	60°C
<b>control</b>	GAPDH	406367	AY818346	60°C
<b>DT of CXCL14</b>	NF-kB	110438511	NW_018394395.1	59°C
	SCX-a	100034489	NC_007130.7	60°C
	Pmp22-b	678607	NC_007123.7	59°C
	Mpz	114417	NC_007113.7	60°C
	Mbp-a	326281	<a href="#">NC_007130.7</a>	59°C
<b>DT of CXCL12</b>	OSR1	450059	NC_007124.7	60°C
	OSR2	550389	<a href="#">NC_007127.7</a>	59°C
	COL17A1b	568794	NC_007124.7	59°C

<b>Primer Design Set #1: Sequence Information</b>				
	<b>Gene</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>	<b>Fragment size</b>
<b>control</b>	B-actin	5-TCACCACCACAGCCGAAAG-3	5-AGAGGCAGCGGTTCCCAT-3	98bp
<b>control</b>	GAPDH	5-GTGTAGGCGTGGACTGTGGT-3	5-TGGGAGTCAACCAGGACAAATA-3	121bp
<b>DT of CXCL14</b>	NF-kB	5-GCTCCAGGATGACGTTTCAGTA-3	5-CCAGAAAGTCCCCTTGAGGT-3	201 bp
	SCX-a	5-TCAGGGAGGGATGAGAGCAG-3	5-TCTGCTCCAGAGAACCGAGA-3	335 bp
	Pmp22-b	5-TGGAAACGAGGAGCAGAACC-3	5-AGTGAACCTGAGAGGAGGGT-3	496 bp
	Mpz	5-ATTGCGTCCTTAGCCCCATC-3	5-GTGTTTGTATCCTCCAGCCTCT-3	177 bp
	Mbp	5-TGTCCGAGTCAAGTTGCTACA-3	5-GCTTTTGGTTGGGCAGTCAG-3	316 bp
<b>DT of CXCL12</b>	OSR1	5-CCTCAATCCCCTGTTCCCC-3	5-AGGCAGGTATGAGCAGGAATG-3	484 bp
	OSR2	5-AGTCTTACTGCCCATTCCTG-3	5-AACCGCTCAAACCTGTGTGTTTC-3	116 bp
	COL17A1b	5-TCACTTTGTCACTAATGCCGAT-3	5-TCACCCTTTTCTCCCCTTGG-3	327 bp

<b>Primer Design Set #2: General Information</b>				
	<b>Gene</b>	<b>Gene ID</b>	<b>Accession #</b>	<b>Annealing temp for PCR amplification</b>
<b>Control</b>	B-actin	57934	AF057040	60°C
<b>Control</b>	GAPDH	406367	AY818346	60°C
<b>Ligands</b>	CXCL14-a	58151	NM_131627	59°C
	CXCL12-a	352944	NM_178307	59°C
<b>Receptors</b>	CXCR4-b	114447	NC_007120.7	58°C
	CXCR7-b	561050	NC_007117.7	59°C
<b>Genes expressed in neuromasts and primordium</b>	Snail-1b	792194	NM_130989.3	60°C
	Cldn-2	562525	NC_007132.7	60°C
	Epcam	406454	NC_007124.7	59°C

<b>Primer Design Set #2: Sequence Information</b>				
	<b>Gene</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>	<b>Fragment size</b>
<b>Control</b>	B-actin	5'-TCACCACCACAGCCGAAAG-3'	5-AGAGGCAGCGTTCCCAT-3	98bp
<b>Control</b>	GAPDH	5'-GTGTAGGCGTGGACTGTGGT-3'	5'-TGGGAGTCAACCAGGACAAATA-3'	121 bp
<b>Ligands</b>	CXCL14-a	5'-TGCAGATGCACAAGAAAAGG-3'	5'-GGCTCAAACGTCCTGTGTT-3'	225 bp
	CXCL12-a	5'-TTCATGCACCGATTTC AAC-3'	5'-TGTTGATGGCGTTCCTCAGG-3'	222 bp
<b>Receptors</b>	CXCR4-b	5'-TACGATGTAAGTTGGCTTGTGA-3'	5'-CTGACTGAGAGGTCGCAAAG-3'	435 bp
	CXCR7-b	5'-GAGTTGCCACCACACAAAGG-3'	5'-TGTGCTTAATTGGTCCCTGC-3'	396 bp
<b>Genes expressed in neuromasts and primordium</b>	Cldn-2	5'-GCGTTTTATTGATTGCAGGCG-3'	5'-TTGCTGCCCGTTTTATGTGC-3'	353 bp
	Snail-1b	5'-CGCTGAAGTTTCGAGGGGAT-3'	5'-CAGTGTTCAGTGGAAAGGC-3'	485 bp
	Epcam	5'-CTGCCCTGTTTTGGAATGG-3'	5'-GCGTTTGAAAACGAGACCTT-3'	375 bp



## Quantitative Real-Time Polymerase Chain Reaction (qPCR)

1. Prepare qPCR reactions
  - a. Thaw 2x GoTaq qPCR Master Mix (Promega Corporation, Madison, WI, USA), primer sets, and cDNA on ice
    - i. Gently vortex and centrifuge 2x qPCR master mix
      1. Avoid foaming or extended exposure to light
  - b. Prepare reaction master mix (MM) for each primer set
    - i. Protocol was adapted to cut reaction mix from 50 ul to 25 ul

Components	9.5x MM	19x MM (for duplicates)	Final concentration
2x qPCR Master Mix	12.5 ul	237.5 ul	1x
Forward Primer	0.25 ul	4.75 ul	0.2 uM or 0.05- 0.9 uM
Reverse Primer	0.25 ul	4.75 ul	0.2 uM or 0.05-0.9 uM
Nuclease-free H <sub>2</sub> O	9.5 ul	180.5 ul	To final volume of 22.5 ul *before cDNA is added
cDNA *add directly to well	2.5 ul	2.5 ul	

- c. Add corresponding cDNA (2.5 ul) to corresponding well in the 96-well plate (Applied Biosystems, Foster City, CA, USA)
      - i. This is a modification made to the protocol. Previously, we used 8-strip qPCR tubes
    - d. Pipette 22.5 ul of the 9.5x MM into each corresponding well (based off primers)
      - i. Careful to avoid air bubbles
    - e. Seal 96-well plate with adhesive cover
      - i. Ensure there are no bubbles or wrinkles present
    - f. Spin 96-well plate in the plate centrifuge (Benchmark Scientific Inc., Sayreville, NJ, USA)
      - i. Ensure there are no air bubbles or side droplets
2. Run qPCR reactions
  - a. Bring 96-well plate to the qPCR machine (Applied Biosystems, Foster City, CA, USA)
    - i. Load 96-well plate inside the qPCR machine
      1. Verify that the loading base is labelled “for 96-well plate use only” and not “for 8-strip tubes”
        - a. This will cause the door to become unaligned

- b. Open the software
  - i. Login to PC computer by qPCR machine
    1. Username: INSTR-USER
    2. Password: INSTR-USER
  - ii. Go to “7500 Software”
    1. Use “Guest” (unless you have an account listed)
    2. Select “continue without connection”
- c. Set up the qPCR reaction
  - i. Under “Experiment Properties”
    1. Select “Design Wizard”
    2. Experiment name: NAME IT! (AC date qPCR)
    3. Which instrument: 7500 (96 wells)
    4. Type of experiment: Quantitation
  - ii. Under “Methods and Materials”
    1. Quantitation Method: Comparative  $C_T$  ( $\Delta\Delta C_T$ )
    2. Which reagents: SYBR Green Reagents
    3. Which ramp speed: Standard (~2 hours to complete run)
    4. Which template: cDNA (complementary DNA)
  - iii. Under “Targets”
    1. How many targets: 1
    2. Reporter: SYBR
    3. Quencher: None
  - iv. Under “Samples”
    1. How many samples: answer varies\*
      - a. If running a qPCR (using both Actin and GAPDH as controls) without replicates: 81
        - i. Troubleshooting: There should be 96 samples. Any left-over wells should be filled with RNase-free  $H_2O$  and used as blanks
      - b. If running a qPCR (using just Actin as a control) without replicates: 72
        - i. Troubleshooting: There should be 96 samples. Any left-over wells should be filled with RNase-free  $H_2O$  and used as blanks
      - c. If running a qPCR with duplicates or triplicate: 96
        - i. Left-over wells were filled with RNase-free  $H_2O$  and used as blanks.

**\* Recommendation: only run reactions in duplicates or triplicates. Load blanks**

  2. How many replicates: 1

3. How many negative controls: 0
4. Arrange plate by: Columns
5. Set up biological replicate groups: No biological replicates
6. When prompted, label the samples according to the order in which they appear and assign a color
  - a. This makes it easier when finding samples for the data analysis
- v. Under “relative quantitation settings”
  1. Which sample as reference sample: sample 1
  2. Which target as endogenous control: target 1
- vi. Under “Run Method”
  1. Reaction volume per well: 25 ul
  2. Ensure accurate reaction stage set up
    - a. Holding Stage
      - i. 95°C for 10:00 minutes
    - b. Cycling stages (40)
      - i. 95°C for 0:15 seconds
      - ii. 60°C for 1:00 minute
    - c. Melting Curve Stages
      - i. 95°C for 0:15 seconds
      - ii. 60°C for 1:00 minute
      - iii. 95°C for 0:30 seconds
      - iv. 60°C for 0:15 seconds
  3. See reaction set up then select “Finish designing experiment”
- vii. Start run
  1. Should be completed after 2 hours and 10 minutes.
- viii. Store qPCR reactions in -20 °C freezer until ready to run gels

### **qPCR Troubleshooting Guide:**

1. Use plate/tube holders. Do not allow tubes or plates to contact the bench, ice, or other surfaces
  - a. We switched to 96-well plates since 8-strip tubes advertised as “qPCR tubes” do not always correspond with the machine
    - i. This happened, causing the qPCR door to be “unaligned”
2. Do not touch the optical surfaces of plates/tubes (the top or bottom)
  - a. Do everything in your power to keep plates dust-, dirt- and fingerprint-free
  - b. If you keep the 96-well plate on ice, the condensation forming can have an impact on the optical readings. Avoid contact with ice.
3. Be sure to load every well
  - a. This keeps the machine balanced as well as providing room for “blanks”

### Original qPCR Trial Set-up

	1	2	3	4	5	6	7	8	9	10	11	12
A	Actin 3	9	8	7	5	6	2	4	1	Snail 3	9	H <sub>2</sub> O
B	1	GAPDH 3	9	8	7	5	6	2	4	1	H <sub>2</sub> O	H <sub>2</sub> O
C	4	1	CXCL14 3	9	8	7	5	6	2	4	H <sub>2</sub> O	H <sub>2</sub> O
D	2	4	1	CXCL12 3	9	8	7	5	6	2	H <sub>2</sub> O	H <sub>2</sub> O
E	6	2	4	1	CXCR4 3	9	8	7	5	6	H <sub>2</sub> O	H <sub>2</sub> O
F	5	6	2	4	1	CXCR7 3	9	8	7	5	H <sub>2</sub> O	H <sub>2</sub> O
G	7	5	6	2	4	1	Epcam 3	9	8	7	H <sub>2</sub> O	H <sub>2</sub> O
H	8	7	5	6	2	4	1	Cldn 3	9	8	H <sub>2</sub> O	H <sub>2</sub> O

### Trial Set-up for Duplicates: qPCR Part A- Receptors and Ligands

	1	2	3	4	5	6	7	8	9	10	11	12
A	Actin 3	9	8	7	5	6	H <sub>2</sub> O	5	6	2	4	1
B	1	CXCL14 3	9	8	7	5	H <sub>2</sub> O	7	5	6	2	4
C	4	1	CXCL12 3	9	8	7	H <sub>2</sub> O	8	7	5	6	2
D	2	4	1	CXCR4 3	9	8	Actin 3	9	8	7	5	6
E	6	2	4	1	CXCR7 3	9	1	CXCL14 3	9	8	7	5
F	5	6	2	4	1	H <sub>2</sub> O	4	1	CXCL12 3	9	8	7
G	7	5	6	2	4	H <sub>2</sub> O	2	4	1	CXCR4 3	9	8
H	8	7	5	6	2	H <sub>2</sub> O	6	2	4	1	CXCR7 3	9

### Trial Set-up for Duplicates: qPCR Part B- Other Transcription Factors

	1	2	3	4	5	6	7	8	9	10	11	12
A	Actin 3	9	8	7	5	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	6	2	4	1
B	1	Epcam 3	9	8	7	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	5	6	2	4
C	4	1	Cldn 3	9	8	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	7	5	6	2
D	2	4	1	Snail 3	9	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	8	7	5	6
E	6	2	4	1	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	Actin 3	9	8	7	5
F	5	6	2	4	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	1	Epcam 3	9	8	7
G	7	5	6	2	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	4	1	Cldn 3	9	8
H	8	7	5	6	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O	2	4	1	Snail 3	9

## Analyzing qPCR Data

1. Export data from qPCR machine onto an excel sheet (Microsoft Corporation, Redmond, WA, USA)
2. Categorize  $C_T$  values by gene
  - a. Create different tabs and label them according to gene name
3. Organize  $C_T$  values by time point
  - i.e. Non-injected 24 hours, Sham-injected 24 hours, and Morpholino-injected 24 hours
4. Keep actin control  $C_T$  values below  $C_T$  values for genes of interest
5. Perform Mann-Whitney tests to analyze data
  - a. Calculate  $\Delta C_T$  values based off  $C_T$  values- See Figure 5
    - i. Subtract the  $C_T$  values for gene of interest samples by control samples
      - i.e. (CXCL12  $C_T$  value for Non-Injected 24 hpf) – (Actin  $C_T$  Non-Injected 24 hpf)
    - ii. Repeat for each trial, time point and injection per gene of interest
    - iii. Remove trials where control values were not characteristic
      - i.e. An actin  $C_T$  value between 25-40

Sample	INJ.	$C_T$	$\Delta C_T$
	TIME POINT		Sample-Actin
<b>CXCL12</b>			
3	NON 24	16.836	5.519
1	SHAM 24	18.158	6.627
4	MO 24	16.413	2.963
2	NON 36	16.192	5.423
6	SHAM 36	16.028	4.358
5	MO 36	16.796	5.241
7	NON 48	16.316	4.921
8	SHAM 48	17.403	5.193
9	MO 48	17.216	5.288
<b>Actin</b>			
3	NON 24	11.317	
1	SHAM 24	11.531	
4	MO 24	13.45	
2	NON 36	10.769	
6	SHAM 36	11.67	
5	MO 36	11.555	
7	NON 48	11.395	
8	SHAM 48	12.21	
9	MO 48	11.928	

**Figure 5: Example for Calculating  $\Delta C_T$  values.** Data were arranged by  $C_T$  values for genes of interest and endogenous control (Actin).  $C_T$  values for experimental samples were subtracted from  $C_T$  values for control samples. Dots denote which numbers are being subtracted from each other to calculate  $\Delta C_T$  values. Repeat this process for all injection incubation periods (non-injections-green, sham injection-red, morpholino injected- blue), trials and genes of interest. Remove any trials where the control  $C_T$  values are uncharacteristically high (between 25-40).

- b. Open data analysis software
    - i. In this case, GraphPad Prism (GraphPad Software, San Diego, CA, USA)
  - c. Find “New table and graph”
    - i. Under “XY,” select “grouped”
      - 1. Grouped tables have two grouping variables, one defined by columns and the other defined by rows
      - 2. Under “Data table,” select “enter data into a new table”
      - 3. Under “options,” select “enter and plot a single Y value for each point.”
      - 4. Click on “create”
  - d. Add calculated  $\Delta C_T$  values for each gene of interest trial to the data sheet
    - i. On the top of the sheet are groups
      - 1. Assign labels based on injection type and incubation period
        - i.e. Group A: Non-Injected 24 hpf, Group B: Sham Injected 24 hpf, Group C: Morpholino Injected 24 hpf
      - a. Repeat for each time point
    - ii. Once data is organized, click “Analyze”
  - e. Log transform data
    - i. Under “Transform, normalize...” select “transform”
    - ii. Under “Parameters,” select “Transform Y values using...”
      - 1. In the drop-down box, find and select “Y= Log2(Y)”
        - a. Click “ok”
  - f. Once data is transformed, click “Analyze”
  - g. Setting up analysis parameters
    - i. Under “Column analyses,” select “t tests (and nonparametric tests)”
      - 1. Select two groups at a time (compare injected to non-injected)
    - ii. Under “Experimental Design,” select “unpaired”
      - 1. Under “assume gaussian distribution?” select “No. Use nonparametric test.”
    - iii. Under “Choose test,” select “Mann-Whitney test. Compare ranks”
    - iv. Hit “ok” to run Mann-Whitney test
  - h. Record p-values
    - i. Repeat process for all genes of interest
      - 1. Compare non-injected to sham injected
        - a. Repeat for each time point
      - 2. Compare non-injected to morpholino injected
        - a. Repeat for each time point
      - 3. Compare sham injected to morpholino injected
        - a. Repeat for each time point
6. For graphing data
- a. Average  $C_T$  values for each injection incubation period and for each gene of interest- See Figure 5

- b. Calculate the standard deviation range for each data set
  - i. Begin by calculating the standard deviation (st. dev) of the data set
  - ii. To find the lowest acceptable value, subtract the calculated standard deviation from the average
  - iii. To find the highest acceptable value, add the calculated standard deviation to the average

\*This analysis can also be completed with 2-standard deviations

- c. Remove any outliers
  - i. Orange cells denote data points that fall outside the standard deviation
  - ii. Yellow cells denote data points within decimal points of the accepted standard deviation range
- d. Re-average data that fall within the acceptable standard deviation range

CXCL12	Sample	TRIAL 1	TRIAL 2	TRIAL 3	TRIAL 4	TRIAL 5	TRIAL 6	TRIAL 7		Re-AVERAGE	Avg	St. Dev	Upper	Lower
12 3	NON 24	19.8197	24.20403	16.3929	27.5248	15.6443	15.4048	16.9214		16.83663826	19.416	4.73493	24.1509	14.68
12 1	SHAM 24	16.3941	16.49703	16.8058	22.5367	38.1166	15.7469	20.9719		18.15874672	21.0099	7.97931	28.9892	13.03
12 4	MO 24	18.3801	14.79802	17.1703	16.8699	17.1719	16.7629	15.7096		16.41377894	16.6947	1.1473	17.842	15.55
12 2	NON 36	15.2611	15.90563	16.5032	17.2004	22.2167	16.72	15.5673		16.19294882	17.0535	2.37464	19.4281	14.68
12 6	SHAM 36	14.3286	15.27522	17.2867	23.3666	15.2102	18.3209	15.7493		16.02848117	17.0768	3.08755	20.1643	13.99
12 5	MO 36	18.1421	17.9244	16.1204	16.2925	14.6693	20.57	15.501		16.79606304	17.0314	1.99374	19.0251	15.04
12 7	NON 48	16.3952	16.84957	15.9118	15.4852	16.109	17.7554	17.6531		16.3164072	16.5942	0.86655	17.4607	15.73
12 8	SHAM 48	29.2721	17.57527	15.4422	15.5925	22.5674	16.2423	16.9994		17.40318378	19.0987	5.10033	24.1991	14.00
12 9	MO 48	18.2281	16.33657	15.9765	17.1018	22.068	18.7697	16.884		17.21610721	17.9092	2.08366	19.9929	15.83

**Figure 6: Example for Averaging C<sub>T</sub> values by trial.** Data were organized by gene of interest, sample type, and trials. All trials were averaged, and standard deviations were calculated. Standard deviations were added to and subtracted from averages to find standard deviation range. Any outliers falling outside of the acceptable standard deviation range were excluded and data were re-averaged. Orange cells denote data points falling outside the acceptable standard deviation range. Yellow cells denote data points within a few decimal points from the accepted standard deviation range.

- e. Calculate  $\Delta C_T$  values based off accepted C<sub>T</sub> values- Figure 7
  - i. Subtract the C<sub>T</sub> values for gene of interest samples from the C<sub>T</sub> values for actin control samples
  - i.e. (CXCL12 C<sub>T</sub> value for Non-Injected 24 hpf) – (Actin C<sub>T</sub> Non-Injected 24 hpf)
  - ii. Repeat for each injection incubation period per gene of interest
  - iii. Also repeat this step for every trial

Sample	INJ.	C <sub>T</sub>	ΔC <sub>T</sub>
	TIME POINT	AVERAGED	Sample-Actin
<b>CXCL12</b>			
3	NON 24 ●	16.836	5.519
1	SHAM 24 ●	18.158	6.627
4	MO 24 ●	16.413	2.963
2	NON 36 ●	16.192	5.423
6	SHAM 36 ●	16.028	4.358
5	MO 36 ●	16.796	5.241
7	NON 48 ●●	16.316	4.921
8	SHAM 48 ●●	17.403	5.193
9	MO 48 ●●	17.216	5.288
<b>Actin</b>			
3	NON 24 ●	11.317	
1	SHAM 24 ●	11.531	
4	MO 24 ●	13.45	
2	NON 36 ●	10.769	
6	SHAM 36 ●	11.67	
5	MO 36 ●	11.555	
7	NON 48 ●●	11.395	
8	SHAM 48 ●●	12.21	
9	MO 48 ●●	11.928	

Sample	INJ.	C <sub>T</sub>	ΔC <sub>T</sub>
	TIME POINT	AVERAGED	Sample-Actin
<b>CXCL12</b>			
3	NON 24 ●	16.836	5.519
1	SHAM 24 ●	18.158	6.627
4	MO 24 ●	16.413	2.963
2	NON 36 ●	16.192	5.423
6	SHAM 36 ●	16.028	4.358
5	MO 36 ●	16.796	5.241
7	NON 48 ●●	16.316	4.921
8	SHAM 48 ●●	17.403	5.193
9	MO 48 ●●	17.216	5.288
<b>Actin</b>			
3	NON 24 ●	11.317	
1	SHAM 24 ●	11.531	
4	MO 24 ●	13.45	
2	NON 36 ●	10.769	
6	SHAM 36 ●	11.67	
5	MO 36 ●	11.555	
7	NON 48 ●●	11.395	
8	SHAM 48 ●●	12.21	
9	MO 48 ●●	11.928	

Sample	INJ.	C <sub>T</sub>	ΔC <sub>T</sub>
	TIME POINT	AVERAGED	Sample-Actin
<b>CXCL12</b>			
3	NON 24 ●	16.836	5.519
1	SHAM 24 ●	18.158	6.627
4	MO 24 ●	16.413	2.963
2	NON 36 ●	16.192	5.423
6	SHAM 36 ●	16.028	4.358
5	MO 36 ●	16.796	5.241
7	NON 48 ●●	16.316	4.921
8	SHAM 48 ●●	17.403	5.193
9	MO 48 ●●	17.216	5.288
<b>Actin</b>			
3	NON 24 ●	11.317	
1	SHAM 24 ●	11.531	
4	MO 24 ●	13.45	
2	NON 36 ●	10.769	
6	SHAM 36 ●	11.67	
5	MO 36 ●	11.555	
7	NON 48 ●●	11.395	
8	SHAM 48 ●●	12.21	
9	MO 48 ●●	11.928	

**Figure 7: Example for calculating ΔC<sub>T</sub> values.** Data were organized by average C<sub>T</sub> values for genes of interest and the endogenous control (β-actin). Averaged C<sub>T</sub> values for experimental samples were subtracted from averaged control C<sub>T</sub> values. Dots denote which numbers are being subtracted from each other to calculate ΔC<sub>T</sub> values. Repeat this process for all injection incubation periods (non-injected- green, sham injected- red, morpholino injected- blue) and genes of interest.

- f. Calculate ΔΔC<sub>T</sub> values- Figure 8
  - i. Comparing sham injected embryos to non-injected embryos
    1. To find ΔΔC<sub>T</sub> values for sham injected embryos, subtract the ΔC<sub>T</sub> values of sham injected samples from the ΔC<sub>T</sub> values for non-injected samples
      - i.e. (CXCL12 ΔC<sub>T</sub> value for sham injected 24 hpf) – (CXCL12 ΔC<sub>T</sub> value for non-injected 24 hpf)
    2. Repeat this process for each time point and for every gene of interest
  - ii. Comparing morpholino injected embryos to non-injected embryos
    1. To find ΔΔC<sub>T</sub> values for morpholino injected embryos, subtract the ΔC<sub>T</sub> values of morpholino injected samples from the ΔC<sub>T</sub> values for non-injected samples
      - i.e. (CXCL12 ΔC<sub>T</sub> value for morpholino injected 24 hpf) – (CXCL12 ΔC<sub>T</sub> value for non-injected 24 hpf)
    2. Repeat this process for each time point and for every gene of interest



- iii. Comparing morpholino injected embryos to sham injected embryos
1. To find  $\Delta\Delta C_T$  values for morpholino injected embryos, subtract the  $\Delta C_T$  values of morpholino injected samples from the  $\Delta C_T$  values for sham injected samples
    - i.e. (CXCL12  $\Delta C_T$  value for morpholino injected 24 hpf) – (CXCL12  $\Delta C_T$  value for sham injected 24 hpf)
  2. Repeat this process for each time point and for every gene of interest

Sample	INJ.	Ct	$\Delta C_T$	$\Delta\Delta C_T$
	TIME POINT	AVERAGED	Sample-Actin	Inj. vs Non-inj
<b>CXCL12</b>				
3	NON 24	16.836	5.519	
1	SHAM 24	18.158	6.627	1.108
4	MO 24	16.413	2.963	-2.556
2	NON 36	16.192	5.423	
6	SHAM 36	16.028	4.358	-1.065
5	MO 36	16.796	5.241	-0.182
7	NON 48	16.316	4.921	
8	SHAM 48	17.403	5.193	0.272
9	MO 48	17.216	5.288	0.367
<b>Actin</b>				
3	NON 24	11.317		
1	SHAM 24	11.531		
4	MO 24	13.45		
2	NON 36	10.769		
6	SHAM 36	11.67		
5	MO 36	11.555		
7	NON 48	11.395		
8	SHAM 48	12.21		
9	MO 48	11.928		

Sample	INJ.	Ct	$\Delta C_T$	$\Delta\Delta C_T$
	TIME POINT	AVERAGED	Sample-Actin	Inj. vs Non-inj
<b>CXCL12</b>				
3	NON 24	16.836	5.519	
1	SHAM 24	18.158	6.627	1.108
4	MO 24	16.413	2.963	-2.556
2	NON 36	16.192	5.423	
6	SHAM 36	16.028	4.358	-1.065
5	MO 36	16.796	5.241	-0.182
7	NON 48	16.316	4.921	
8	SHAM 48	17.403	5.193	0.272
9	MO 48	17.216	5.288	0.367
<b>Actin</b>				
3	NON 24	11.317		
1	SHAM 24	11.531		
4	MO 24	13.45		
2	NON 36	10.769		
6	SHAM 36	11.67		
5	MO 36	11.555		
7	NON 48	11.395		
8	SHAM 48	12.21		
9	MO 48	11.928		

Sample	INJ.	Ct	$\Delta C_T$	$\Delta\Delta C_T$
	TIME POINT	AVERAGED	Sample-Actin	MO vs Sham
<b>CXCL12</b>				
3	NON 24	16.836	5.519	
1	SHAM 24	18.158	6.627	
4	MO 24	16.413	2.963	-3.664
2	NON 36	16.192	5.423	
6	SHAM 36	16.028	4.358	
5	MO 36	16.796	5.241	0.883
7	NON 48	16.316	4.921	
8	SHAM 48	17.403	5.193	
9	MO 48	17.216	5.288	0.095
<b>Actin</b>				
3	NON 24	11.317		
1	SHAM 24	11.531		
4	MO 24	13.45		
2	NON 36	10.769		
6	SHAM 36	11.67		
5	MO 36	11.555		
7	NON 48	11.395		
8	SHAM 48	12.21		
9	MO 48	11.928		


**Figure 8: Example for Calculating  $\Delta\Delta C_T$  values.** To calculate the  $\Delta\Delta C_T$  values between non-injected and injected samples, non-injected  $\Delta C_T$  values were subtracted from either sham injected (red)  $\Delta C_T$  values or morpholino injected (blue)  $\Delta C_T$  values. To calculate the  $\Delta\Delta C_T$  values between injections (purple), sham injected  $\Delta C_T$  values were subtracted from morpholino injected  $\Delta C_T$  values. Highlighted cells denote calculated  $\Delta\Delta C_T$  values. Repeat this process for all genes of interest.



- g. Calculate Relative quantification (RQ)- see Figure 10
- i. Follow the equation:  $2^{(-\Delta\Delta C_T)}$
  - ii. This will give you folding values for each of your samples

Sample	INJ.	Ct	$\Delta C_T$	$\Delta\Delta C_T$	$\Delta\Delta C_T$	Folding Value	Folding Value
	TIME POINT	AVERAGED	Sample-Actin	Inj. vs Non-inj	MO vs Sham	$2^{-(\Delta\Delta C_T)}$	$2^{-(\Delta\Delta C_T)}$
<b>CXCL12</b>						non vs inj.	between inj.
3	NON 24	16.836	5.519			1	
1	SHAM 24	18.158	6.627	1.108		0.463936738	1
4	MO 24	16.413	2.963	-2.556	-3.664	5.880749346	12.67575697
2	NON 36	16.192	5.423			1	
6	SHAM 36	16.028	4.358	-1.065		2.09216988	1
5	MO 36	16.796	5.241	-0.182	0.883	1.134455485	0.542238704
7	NON 48	16.316	4.921			1	
8	SHAM 48	17.403	5.193	0.272		0.828170661	1
9	MO 48	17.216	5.288	0.367	0.095	0.775393206	0.936272247
<b>Actin</b>							
3	NON 24	11.317					
1	SHAM 24	11.531					
4	MO 24	13.45					
2	NON 36	10.769					
6	SHAM 36	11.67					
5	MO 36	11.555					
7	NON 48	11.395					
8	SHAM 48	12.21					
9	MO 48	11.928					

**Figure 9: Example for Calculating RQ values.** To compare injected samples (sham injected- red and morpholino injected- blue) to non-injected samples (green), folding values were calculated using  $\Delta\Delta C_T$  values labelled “injected vs non-injected” and used in the formula:  $2^{-\Delta\Delta C_T}$ . To compare sham injected samples (red) to morpholino injected samples (blue), folding values were calculated using the  $\Delta\Delta C_T$  values labelled “between injections” and used in the formula:  $2^{-\Delta\Delta C_T}$ . Repeat this process for all genes of interest.

- h. Graph folding values using Excel
  - i. Go to “Insert” and select “Insert Column or Bar graph”
    1. When prompted, select 2-D Clustered Column
  - ii. Go to “Chart Tools” and select “Design”
    1. Under “Design,” click on “Select Data”
  - iii. Go to “Legend Entries Series” and select “Add”
    1. When prompted, label “Series Name” as Insert Gene of Interest Name Here Folding Values
      - i.e. Series Name: CXCL12 Folding Values
  - iv. For “Series values,” highlight the calculated folding values
    1. Select “ok”
  - v. Go to “Horizontal (Category) Axis Labels” and select “Edit”
    1. For “Axis Label Range,” select “Injections by Time Point” labels
    2. Select “ok”

- vi. Go to “Chart Tools” and select “Design”
    - 1. Under “Design,” click on “Add Chart Element”
      - a. Find “Axes” and select “More Axis Options”
        - i. Under “Format Axis,” click “Axis Options”
          - 1. Select “Vertical (Value) Axis”
          - 2. Click this button 
            - a. Click on “Axis Options”
              - i. Select “Logarithmic scale”
              - ii. Edit Base number to “2”
      - b. Find “Axis Titles” and select both “Horizontal” and “Vertical” axis
        - i. Label the horizontal axis “Injection and Incubation Period”
        - ii. Label the vertical axis “Relative Gene Expression”
- vii. Repeat this process for:
  - 1. All genes of interest → Non-injected, sham injected, and morpholino injected at 24 hpf
  - 2. All genes of interest → Non-injected, sham injected, and morpholino injected at 36 hpf
  - 3. All genes of interest → Non-injected, sham injected, and morpholino injected at 48 hpf
  - 4. All genes of interest → sham injected and morpholino injected at 24 hpf
  - 5. All genes of interest → sham injected and morpholino injected at 36 hpf
  - 6. All genes of interest → sham injected and morpholino injected at 48 hpf
- i. Graphing folding values using GraphPad PRISM
  - i. Open GraphPad Prism (GraphPad Software, San Diego, CA, USA)
  - ii. Find “New table and graph”
    - 1. Under “XY,” select “grouped”
      - a. Grouped tables have two grouping variables, one defined by columns and the other defined by rows
      - b. Under “Data table,” select “enter data into a new table”
      - c. Under “options,” select “enter and plot a single Y value for each point”
  - iii. Add calculated folding values from Excel sheet
    - 1. On the left side of the GraphPad data sheet are numbers
      - a. Assign labels based on time points- hours post-fertilization (hpf)
        - i.e. 1: 24 hpf, 2: 36 hpf, 3: 48 hpf
- iv. On the top of the sheet are groups

1. Assign labels based on injection type
  - i.e. Group A: Non-Injected, Group B: Sham Injected, Group C: Morpholino Injected
2. Organized data according to the labels
- v. Go to the Navigator Panel under “Graphs”
  1. Select “New Graph”
    - a. Under “Table,” select the Data sheet you want to graph
    - b. Under “Kind of Graph,” select “Grouped”
    - c. Find “Summary data” and select 
- vi. To edit graph
  1. Find “Change” and select 
    - a. Under “Left Y axis,” change scale to “Log 2”
      - i. Click on “ok”
  2. Label the X-axis and Y-axis
  3. Title graph
- vii. To add error bars and averages
  1. Select the data table
  2. Find “Table format: grouped” and select “enter and plot error values calculated elsewhere”
    - a. In the dropdown box, select “Mean, SD, N”
      - i. Add values to the data table

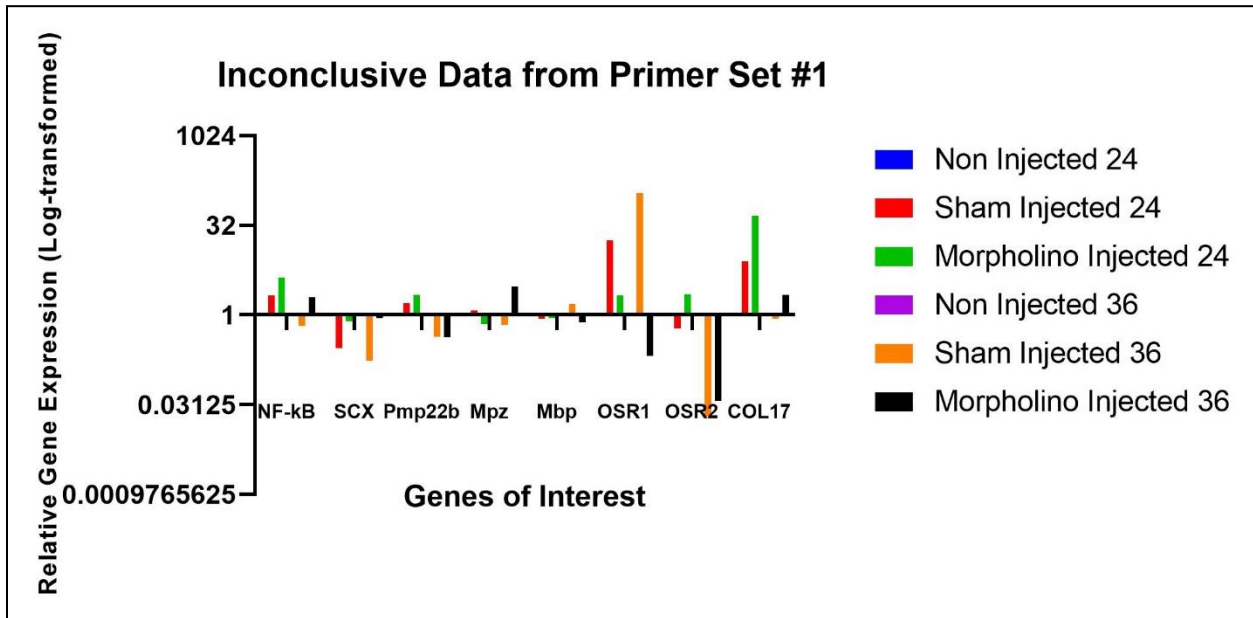
## How to Make, Run, and Visualize Gels

1. Prepare 1x TAE solution
  - a. If there isn't any made, pour 100 mL of 10X TAE and 900 mL dI H<sub>2</sub>O into a 1L flask
2. Prepare 1% agarose
  - a. Mass 0.40 g of agarose (mass on tared weigh paper)
  - b. Transfer agarose to 125 mL Erlenmeyer flask
  - c. Add 40 mL of 1x TAE to flask with agarose and mix
  - d. Microwave for 30 seconds or until solution bubbles
  - e. Stir solution
  - f. Microwave solution two more times for another 10 seconds
  - g. Stir solution and allow flask to cool
3. Prepare gel tray
  - a. Add 12-well gel comb to designated area in gel tray
  - b. Lift sides of gel tray and screw tightly
  - c. Once it is warm to the touch, pour 1% agarose into gel tray
  - d. Allow gel to set for roughly 15 minutes
4. Prepare gel electrophoresis box (Fotodyne Inc., Hartland, WI, USA)
  - a. Once 1% agarose gel is set, remove gel comb, unscrew the sides of the gel tray, push sides down and screw tightly
  - b. Place gel tray in electrophoresis box
    - i. Make sure wells are near the black lead end of the gel box
  - c. Add 1x TAE to gel box, allowing it to cover the gel
5. Load samples
  - a. When running an RNA gel, load 15 ul of RNA with 2 ul loading dye
  - b. When running qPCR gels, load 15 ul of qPCR sample with 2 ul loading dye
  - c. Always load at least one lane with 8 ul of DNA marker with 2 ul loading dye
  - d. Load samples one at a time through the buffer
    - i. Be sure not to puncture the gel with pipette tips

\* Run one gene of interest (9 samples and marker) per gel
6. Run gel
  - a. Connect leads (black with black, red with red)
    - i. Make sure the gel is running towards the red lead
  - b. Set power supply to ~100 Volts constant current for roughly 30-45 minutes
    - i. Make sure dye fronts have travelled 2/3rds down the gel
7. Visualize gel
  - a. Turn on camera and computer
    - i. Log in- Username: calderoa Password: BcLU2019
  - b. Place gel on the UV transilluminator (Fotodyne Inc., Hartland, WI, USA)
  - c. Place hood over transilluminator and turn on UV light

- d. Zoom in on gel
- e. Flip switch to “O” position
- f. Take picture and turn off UV light
- g. Flip switch to “I” position
- h. Camera program should open on the computer
- i. Import picture
- j. Print it with the Mitsubishi printer (Mitsubishi Electric, Tokyo, JP)
  - i. Make sure pixel size is 1280 x 1024
  - ii. Name file: Nancy AC, date, gel type
- k. Log out and turn camera off
- l. Discard gel in appropriate gel container
  - i. Fresh 1% agarose gel container

Appendix V. Inconclusive data from primer set #1



**Figure 10. Effect of Sham and Morpholino injections on Gene Expression in *Danio rerio* embryos at 24 and 36 hpf.** RT-qPCR examined the mRNA levels of eight zebrafish genes: *NF-kB*, *SCX*, *Pmp22b*, *mpz*, *mbp*, *OSR1*, *OSR2*, and *COL17* expressed at 24 and 36 hours post-fertilization (hpf) following microinjection with either 1 nl of phenol red (sham) or CXCL14 antisense morpholino in phenol red. Relative gene expression levels were determined using the  $2^{-\Delta\Delta C_t}$  method with  $\beta$ -actin as the reference gene. The statistical significance of the data could not be determined given low sample size and inconclusive results.