A Fundamental Investigation into the Presence of Interstitial Cells of Cajal within the Gastrointestinal Tract of Dania rerio

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A Fundamental Investigation into the Presence of Interstitial Cells of Cajal within the Gastrointestinal Tract of Danio rerio

A Thesis of Department of Biological Sciences

State University of New York College at Brockport

Brockport, New York

By Scott Leddon
# THESIS DEFENSE

Scott Leddon

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Dedication

This thesis is dedicated to all the special people in my life, which have kindled my interest in science and support my scientific endeavors. Thank you all.

Christina Alello, you have shown me that a little touch of crazy can help us all survive in this insane world. Jolene Beckman, thank you for teaching me the smallest whispers can carry the loudest messages. Jake Conner, you have shown that the greatest knowledge that humans have is not our collective intellect, and is in deed our innate humility. Helen Chrispen, you have shown me that wisdom is not a subject in school, but is a process of continued observation in life. Meghan Doherty, from you I have learned of loyalty, there has never been a day that I have felt I could not count on you. Jessica Gallagher of all of my confidantes, you have been perhaps my most endearing. You have been a role model to me in many different facets. You have shown me the values of honesty, hard work, dedication, and perhaps most important friendship. Brianna Hill, you have shown me the value of life, each and every life. Also, you have given me striking
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General Abstract

Coordinated gastrointestinal (GI) motility results from the complex interactions between interstitial cells of Cajal (ICC), enteric neurons, and smooth muscle cells. Kit positive immunoreactivity has been extensively utilized as a marker for ICC; these cells function to generate rhythmic depolarization in the GI smooth muscle termed the electrical slow wave. Coordinated GI motility is regulated by the electrical slow wave. Directly lesioning ICC populations with neutralizing antibodies results in GI dysmotility and loss of the electrical slow wave. Furthermore, GI dysmotility symptoms and other human pathologies have been correlated with ICC deficiencies within the GI tract.

Currently, there are few treatments and therapeutic interventions for GI motility disorders. Irritable Bowl Syndrome alone affects up to 20% of the world population.\textsuperscript{1-5} The high prevalence of GI motility disorders combined with the lack of effective interventions, suggest new treatments are needed. Rational drug development and new treatment design will benefit from the development of new model systems. Greater understanding of the regulation of GI motility, pathologies that affect GI motility, and the development of new therapeutics will all benefit from a new model of GI motility.
This thesis provides support to the use of *Danio rerio*, the zebrafish, as a model of human GI motility. *Danio rerio* is an excellent model system for several human physiological systems or diseases including macular degeneration, neural degenerative diseases, cardiovascular disease, cholesterol and lipid metabolism, and cardiovascular disease.\(^6-20\)

The zebrafish model system offers the opportunity for forward genetic analysis; novel genes, genetic elements, and pathologies can be characterized with reverse genetic screens through the evaluation of interesting phenotypes. Zebrafish reach sexual maturity quickly (three months), produce large numbers of offspring (100 per week), and are more cost efficient to house compared to mammalian models. Most seductive of zebrafish traits, optical transparency of larvae, enables direct visualization in-vivo of organ function (including the GI tract) under physiological settings, without perturbing the organism.

This thesis critically examines the hypothesis that GI motility in the zebrafish involves ICC. Prior to the data presented within this thesis, there have not been any published reports on the presence of ICC within the zebrafish GI tract. Results from this investigation show Kit-like immunoreactivity within the muscular layers of the zebrafish GI tract. Additionally, orthologs for the human receptor tyrosine kinase
Kit, *kita* and *kitb*, and orthologs for the human Kit ligand, *kitla* and *kitlb*, are shown to be expressed within the zebrafish GI tract. Kit receptor and ligand are necessary for ICC development and maintenance in mammals. Expression of *kit* receptors and ligands within the zebrafish GI tract are consistent with the hypothesis that ICC support spontaneous rhythmic contractions in the zebrafish—this suggests that the zebrafish may be a suitable model for GI motility.
Works Cited


General Introduction

The gastrointestinal (GI) tract of higher vertebrates is specialized to digest and absorb nutrients for cellular metabolism. The GI tract is organized in concentric rings of tissue, each with specialized functions. Food is chemically and mechanically digested in the luminal space; this space is at the center of the concentric rings of tissue that make up the GI tract. The four primary layers of the digestive tract, moving from the lumen to the interior of the body, are the mucosa, submucosa, muscularis externa, and the serosa. The anatomy and the function of these layers will be briefly described (also see figure 1 and figure 2).

After food is broken-down into "absorbable" units it is transported into the mucosa via active and passive transport mechanisms. The mucosa consists of the mucosal epithelium, an underlying layer of connective tissue termed the lamina propria, and a thin layer of smooth muscles termed the muscular mucosa. The mucosal epithelium is a complete barrier separating luminal contents from the body, and consists of enterocytes (absorptive cells), goblet cells that secrete mucins, M-cells that transport pathogens to the immune system, and exocrine cells that secrete...
Figure 1. 20X transverse section of the zebrafish GI tract stained with hematoxylin and eosin.
**Figure 2.** 40X transverse section of the zebrafish GI tract stained with hematoxylin and eosin.
fluid and digestive enzymes into the luminal space. Moving from the mucosa towards the muscular layer of the GI tract, the lamina propria is a layer connective tissue that consists of blood vessels, neural processes, immune cells, and blunt ended lymph ducts (lacteals). A thin layer of smooth muscle cells, the muscularis mucosa, underlies the lamina propria and functions to mix and churn the luminal contents, enhancing absorption. The mucosa, consisting of mucosal epithelium, the lamina propria, and the muscular mucosa, is organized into finger-like structures, villi, that project into the luminal space. Absorbed nutrients are transported from entrocytes to veins in the lamina propria, which empty into the hepatic portal vein. Thus, molecules absorbed by the GI system travel directly to the liver for detoxification, and subsequently enter the systemic circulation.

The submucosa is a relatively thick layer of connective tissue containing blood vessels, lymphatic vessels, and immune cells. The muscularis externa is positioned interior to the submucosa, and consists of an inner circular and an outer longitudinal muscular layer. Rhythmic muscular contraction, motility, is partially controlled by the enteric nervous system with cell bodies located within the submucosal and the myenteric plexus. The submucosal plexus separates the circular muscle layer from the submucosa, and the myenteric plexus separates the circular from the longitudinal muscle layers. Interstitial cells of Cajal (ICC) and enteric neurons populate each plexus and
function together to coordinate GI smooth muscle contractions. Effective and efficient motility results from complex motor patterns developed by enteric neurons and by ICC. These coordinated contractions, referred to as GI motility, can be classified as propulsive or mixing. Motility displays circadian rhythms, and is different in the fasted and the fed (postprandial) states. During the fasting state powerful waves of contraction propagate aborally to 'cleanse' the bowel, preventing microbial overpopulation. The outermost layer, the serosa (or adventitia) is a connective tissue layer that serves to hold the GI tract in place within the abdominal-pelvic cavity. The serosa protects the GI tract, supports fragile blood vessels, afferent sensory and efferent autonomic nerves, and lymphatic ducts.

Three basic cell types, often termed the intestinal triad, are necessary for proper GI motility: smooth muscle, enteric neurons, and ICC. Smooth muscle cells (circular and longitudinal) are the force generating, contractile cells, necessary for intestinal motility. Contractions of longitudinal smooth muscle cells shorten GI tract length, while circular smooth muscle contraction narrows the lumen, resulting in segmentation. Stretch receptors activated by mechanical stimuli and chemical receptors monitor luminal content, and transmit information to the enteric nervous system. The peripheral autonomic nervous system also influences the enteric nervous system via the parasympathetic neurotransmitter acetylcholine and
sympathetic neurotransmitter epinephrine. It is important to recognize that neurotransmitters also influence ICC activity, and that ICC integrate and amplify signals from the enteric nervous system. Spontaneous and rhythmic activity occurs in the GI tract, and results in regular waves of contractions that propel the contents of the gut in an aboral direction. ICC are required for ordered propulsion. A change in the resting membrane potential of ICC and smooth muscle cells, termed the electrical slow wave, is correlated with regular muscular contraction. ICC regulate the frequency and strength of these contractions, and form a network within the myenteric plexus region, which functions to distribute and propagate neural input along the length of the GI tract. Clinical dysmotility in humans, such as constipation, Hirschsprung’s disease, intestinal pseudoobstruction, as well as diabetic gastroparesis are correlated with reduced numbers of ICC and/or enteric neurons.

Treatment options for individuals with GI dysmotility are very limited, yet up to 20% of the world population has just one classification of GI dysmotility, Irritable Bowl Syndrome. However, GI dysmotility remains a large unmet medical problem, and represents a huge opportunity for the pharmaceutical industry. In the face of opportunity, GI motility research still poses several very difficult barriers to drug discovery. For example, an intact intestinal triad would ideally be used to screen potential compounds, but this is experimentally difficult.
Merely taking the GI tract out of an animal disturbs the GI tract, causing gastroparesis and inflammation. Cell culture of the complete intestinal triad is challenging, and ICC undergo morphological changes in culture. The paucity of treatment options and success in drug discovery for GI dysmotility therapeutics suggest new model systems are needed.

The zebrafish has many advantages over previous models utilized in GI drug discovery. Zebrafish have been traditionally used as a developmental model because larvae are optically transparent and development occurs quickly. The GI tract of zebrafish larvae can be observed with light microscopy, which allows for the observation of the entire GI system in a non-perturbed state. Additional advantages of the zebrafish model are as follows: the zebrafish can absorb small drug molecules transdermally, which may enable high-throughput-automated screening of combinatorial libraries; transgenic zebrafish are relatively easy to generate due to external fertilization and development of embryos; random mutagenesis followed by phenotype screening can be used to identify novel genes; fecundity is high with 70-300 offspring per pair of zebrafish; and zebrafish colonies are very inexpensive to maintain.

The first step to develop a zebrafish model for GI motility is to determine if the basic cellular components (ICC, enteric neurons, and smooth muscle) are present within the GI tract of the zebrafish, and if these components display similar anatomical
organization to the human GI tract. Preliminary data, generated by Dr. Adam Rich, indicated Kit-like immunoreactivity within whole mount preparations of adult and larvae (7dpf) zebrafish GI tracts. The Kit-like immunoreactivity localized to the GI tract and appeared to be organized in a network. Additionally, kita message of expected size was amplified from GI tissue via reverse transcriptase PCR; these data suggest the presence of ICC within the zebrafish GI tract. Kit immunoreactivity is the standard marker for identification of ICC. However, anatomical localization of ICC within the zebrafish GI tract or their relationship to enteric neurons and smooth muscle has not been established. It is possible that the Kit-like immunohistochemistry localized to a novel zebrafish protein correlating to a novel cell type. Therefore, it is important to verify preliminary data and to show the anatomic position of Kit-positive cells in relation to smooth muscle and enteric neurons.

The primary objective of this thesis is to determine if Kit-positive pacemaker cells, putative interstitial cells of Cajal, are present within the tunica muscularis of the zebrafish GI tract. Expression of kit genes within the tunica muscularis of the GI tract will be examined using anti-Kit antibodies. No zebrafish-specific anti-Kit antibodies are available; therefore, several commercially available anti-Kit antibodies will be examined. These antibodies have been raised against human Kit, mouse Kit, or synthetic peptides with sequences based on human
Kit. Expression of zebrafish \textit{kit} genes will also be examined at the mRNA level. Specific expression of the zebrafish orthologues of human Kit, \textit{kita} and \textit{kitb}, as well as the Kit ligand (Steel factor), \textit{kitla} and \textit{kitlb}, will be determined in GI tissues; these experiments will complement the antibody experiments, and will test for the presence of \textit{kit} expression within the zebrafish GI tract independent of antibody staining. Independent corroboration of \textit{kit} expression is necessary because of the possibility that the anti-Kit antibody could identify an antigen unrelated to Kit. Kit-positive cells within the tunica muscularis of the zebrafish GI tract are expected to localize to an intermediary position, juxtaposed between enteric neurons and smooth muscles of the GI tract, the localization of human GI tract ICC. Anti-Kit antibodies and anti-neuronal or anti-smooth muscle antibodies will be used in dual staining experiments to define anatomical localizations.

Several techniques were developed for experiments within this thesis. First and foremost, an antigen retrieval technique was developed to optimize \textit{kit} antibody labeling. This was necessary because initial attempts showed inconsistent results and poor labeling. This technique enhanced \textit{kit} labeling and was used for all sectioned tissues; these results are given in Chapter 1: Antigen Retrieval. Immunohistochemical analysis of transverse sections required paraformaldehyde fixation, paraffin embedding, and transverse sectioning of GI tissues. Transverse
sections are well-suited to determine the anatomical orientation of Kit-positive cells with respect to layers of smooth muscle and enteric neurons. The simultaneous application of two primary antibodies, termed dual labeling, revealed the precise organization of Kit-positive cells and smooth muscles or enteric neurons in transverse tissue sections; these results are given in Chapter 2: Immunohistochemistry. If the anti-Kit antibody specifically recognizes the zebrafish kit proteins, mRNA for the kit genes should be expressed within zebrafish GI tissues. Reverse transcriptase polymerase chain reaction (RT-PCR) was utilized to identify two zebrafish Kit orthologues, kita and kitb, as well as the ligands for these receptors, kitla (kit ligand a) and kitlb (kit ligand b); these results are given in Chapter 3: Molecular Biology.

The data presented in this thesis show that cells with Kit-like immunoreactivity are located within the tunica muscularis of the zebrafish GI tract, and that these cells are positioned in the myenteric plexus region, intercalated between enteric neurons and smooth muscle cells. Also, GI specific expression of the zebrafish kita, kitb, kitla, and kitlb genes are shown. Taken together these data strongly support the presence of ICC within the muscular layers of the zebrafish GI tract.
Works Cited


General Experimental Approach and

Rationale

Cross-sections of the zebrafish allow for simultaneous-unobstructed visualization of each layer of the GI tract. The anatomical location of the intestinal triad within the zebrafish GI tract was determined using transverse sections immunostained with antibodies specific to Kit (human/mouse ICC marker), desmin (smooth muscle marker), SM-22 (smooth muscle marker), α-tubulin (neuronal process marker), and HU-C/D (neuronal cell body marker).

Initial staining of paraffin-embedded sections with the anti-Kit antibody showed punctuate and non-continuous staining. However, whole mount staining of tissue with the anti-Kit antibody was more consistent. These results indicated that immunostaining artifacts were introduced by the paraffin-embedding and sectioning. A literature search showed that this problem is common in routine immunohistochemistry. Antigen retrieval, a technique that 'unmasks' antigens that have been negatively affected by paraffin-embedding, was applied to sections prior to immunostaining to restore immunoreactivity of the anti-Kit antibody target. The results were more consistent.
staining with the anti-Kit antibody. Smooth muscle and neuronal staining patterns were not affected by the retrieval method; this advance instigated ubiquitous use of antigen retrieval on all paraffin-embedded sections prior to immunostaining.

Single staining with anti-Kit antibody indicated two distinct cell layers with Kit-like immunoreactivity within the GI tract of the zebrafish. To determine if observed Kit-like immunoreactivity was localized to the myenteric plexus region, smooth muscle stains were used in concert with Kit-like staining. Both desmin and SM-22 staining define two layers of smooth muscle, but desmin appears to stain with higher resolution. Dual staining for desmin and Kit revealed Kit-like immunoreactivity localized between the two layers of smooth muscle (myenteric plexus region). Additionally, a second layer of Kit-like immunoreactivity colocalized with circular muscle cells adjacent to the myenteric plexus region, indicating a layer of ICC within the deep muscular plexus (similar to the human colon).

Neuronal staining of cross-sections of the zebrafish GI tract indicated a single layer of neuronal cell bodies and three distinct layers of neuronal processes. Cell bodies of enteric neurons localized to the myenteric plexus region and localized very close to Kit-like immunoreactivity in dual staining experiments. Neuronal processes localized to the myenteric plexus region, the deep muscular plexus region, and also to the lacteal region of the mucosal layer of the GI tract. The neural
process staining was closely associated with Kit-like immunoreactivity in the all regions with the exception of the lacteal region.

Whole mount sample corroborated sectioned tissue data; Dual staining with anti-Kit and anti-neuron antibodies indicated that Kit-like immunoreactivity closely associated, but did not colocalize to the same cell type. Staining with anti-Kit and anti-smooth muscle antibodies showed that Kit-like immunoreactivity occurred strongly between the circular and longitudinal smooth muscle layers (the myenteric plexus), and also indicated more subtle Kit-like immunoreactivity tangential to the circular smooth muscle/myenteric plexus border region (deep muscular plexus). Additionally, whole mount samples indicated that cells within the myenteric plexus region cells displaying Kit-like reactivity were of classical cellular morphology for myenteric ICC (stellate shaped cells with wide cell bodies and multi polar processes). Cells displaying Kit-like immunoreactivity within the deep muscular plexus also displayed classical cellular morphology of deep muscular ICC (slender bi polar cells, with distinct ovular cell bodies).

To support the notion that Kit-like reactivity is recognizing zebrafish \textit{kit} receptors and, thus, ICC populations, the ligands for the \textit{kit} receptors were identified in adult zebrafish GI tissue. A reverse transcriptase PCR approach using RNA isolated for GI tissues as template was utilized to determine
expression of kital and kitbl, co-orthologous pairs of the Steel factor, the mammalian Kit ligand; PCR products were sequenced for verification. The results of these experiments showed that the factors necessary for the stimulation of the kit receptors are expressed within zebrafish GI tissues.

In summary, Kit-like immunoreactivity was observed within the tunica muscularis of the zebrafish GI tract, with localization similar to ICC populations within mammalian and human GI tracts. Kit-like immunoreactivity was closely associated with neuronal staining of cell bodies and processes, which is also consistent with mammalian and humans GI tract organization. The close association of ICC and neurons suggests a conserved structure-function relationship; Thus, ICC within the zebrafish GI tract may function as integrators of neural information for pacemaking regulation of smooth muscle cells. ICC require Kit ligand for development and maintenance in the mouse model; enteric neurons within the mouse GI tract may be an important source for Kit ligand. Immunohistochemical localization of ICC is supported by reverse transcriptase PCR, which detected the presence of mRNA coding for the kit receptors and for the kit ligands; both factors are necessary for ICC development and maintenance in other model systems. Data presented in this thesis supports the existence of ICC within the zebrafish GI tract, and supports ICC within the zebrafish GI tract as regulators of coordinated GI motility; the data
presented in this thesis supports the zebrafish as a complete and important model system for human GI motility.
Materials and Methods

1. Introduction

Detailed methods of each procedure used in this thesis are outlined in Appendix 1: Protocols. Within this section, research approaches will be detailed and specific procedures, Appendix 1: Protocols, will be cited. To complement Appendix 1: Protocols, this section will be broken up into related methods to aid clarity and transparency of this investigation. Protocols describing solution and media preparation will be separate from general protocols.

2. Whole fish, larvae, or adult tissues to thin paraffin tissue sections

This section contains an overview of the procedures necessary to process adult or larva zebrafish into thin sections of paraffin-embedded tissue. This process includes: euthanizing and dissection of fish, fixation and decalcification of samples, tissue processing and embedding, and finally tissue sectioning (Flowchart 1).
Figure 1. Whole fish, larvae, or adult tissues to thin paraffin tissue sections
3. Immunohistochemistry

The overview of protocols in this section will deal with the staining of tissues with antibodies to localize proteins of interest. This overview assumes that thin sectioned tissues are mounted on charged glass slides, and whole mount tissues have not been previously fixed.

The choice of short or long immunostaining protocol does not alter staining intensity or specificity; time restraints are the largest influencing factor when deciding between long or short immunostaining protocols.

The use of antigen retrieval is optional for immunostaining, it may improve or have deleterious effects on immunostaining staining; thus, it is important to test a battery of different antigen retrieval solutions, and only use antigen retrieval if immunostaining on non-retrieved samples fails repeatedly. Details on antigen retrieval are discussed in Chapter One: Antigen Retrieval.
Figure 2. Immunohistochemistry

Type of tissue to be immunostained

Whole mount adult tissue
- Anestize fish in 1X MESAB Protocol 1.4
  - Fix in 4% paraformaldehyde Protocol 1.5
  - Dissect tissue from fish
  - Antigen revival if necessary Protocol 2.15
  - Immunostaining Protocols 2.16 or 2.17
  - Mount samples as desired
  - Image with appropriate fluorescents

Paraffin sections
- Deparaffinize tissue Protocol 2.14
  - Antigen revival if necessary Protocol 2.15
  - Immunostaining Protocols 2.16 or 2.17
  - Mount samples as desired
  - Image with appropriate fluorescents

Whole larvae
- Anestize fish in 1X MESAB Protocol 1.4
  - Fix in 4% paraformaldehyde Protocol 1.6
  - Peel skin from larvae (if not needed) to aid penetration of antibodies
  - Antigen revival if necessary Protocol 2.15
  - Immunostaining Protocols 2.16 or 2.17
  - Mount samples as desired
  - Image with appropriate fluorescents
4. RNA Isolation to cDNA

This section will provide an overview of the procedures necessary to generate cDNA from tissue samples. RNA isolation, RNA purity measurements, RNA concentration measurements, RNA integrity measurements, and first strand synthesis will be addressed; the combination of these procedures will yield high quality DNA for downstream applications.
Figure 3. RNA isolation to cDNA

RNA isolation to cDNA

Isolate total RNA from tissue with RNeasy mini kit (Qiagen) Protocol 3.6

Check RNA integrity by electrophoresis of total RNA on formaldehyde gel. Strong 28s, 26s, and 18s ribosomal RNA bands are expected from proper RNA isolation. Protocol 3.7

Check RNA concentration or yield by UV-spectrophotometry. Protocol 3.8

Check RNA purity by UV-spectrophotometry. Protocol 3.9

5. PCR Protocols

This section will cover protocols necessary to optimize PCR conditions of novel primer sets. Additionally, general procedures for PCR will be addressed. These procedures include both high fidelity DNA polymerase PCR, such as PFX (Invitrogen), utilized for cloning, and Supermix (Invitrogen) PCR protocols, utilized for confirmation of DNA sequences based on product size.
Figure 4. PCR Protocols

- **Primer optimization**
  - Reconstitute lyophilized PCR primers.
    Protocol 4.4
  - Optimize annealing temperature of PCR primers.
    Protocol 4.5
  - Analyze results by agarose gel electrophoresis.
    Protocol 4.6
  - Utilize optimal annealing temperatures, optimize Mg²⁺ concentrations in PCR reaction.
    Protocol 4.7
  - Analyze results by agarose gel electrophoresis.
    Protocol 4.6

- **PFX PCR**
  - Utilize optimal annealing temperatures, optimize Mg²⁺ concentrations in PFX PCR reaction.
    Protocol 4.8
  - Analyze results by agarose gel electrophoresis.
    Protocol 4.6

- **Supermix PCR**
  - Utilize optimal annealing temperatures, optimize Mg²⁺ concentrations in Supermix PCR reaction.
    Protocol 4.9
  - Analyze results by agarose gel electrophoresis.
    Protocol 4.6
6. DNA Sequencing

This section contains an overview of the direct sequencing of PCR products, and the sequencing of PCR products that have been subcloned into vectors. This section assumes that gene specific PFX PCR has been completed, specificity of PCR has been confirmed through agarose gel electrophoresis, and PCR sample has been reserved for sequencing. Finally, this section assumes that sample will be sent to Cornell Biotechnology Center (Ithaca, NY) for sequencing.
Figure 5. DNA Sequencing

DNA Sequencing

Sequencing PCR products

Multiple bands are seen after electrophoretic separation of PCR reaction

- Clean PCR products using mini elute spin columns (Qiagen). Protocol 5.5
- Measure DNA concentration or yield by UV-spectrophotometry. Protocol 5.7
- Measure DNA purity by UV-spectrophotometry. Protocol 5.8
- Prepare PCR sequencing reaction. Protocol 5.9
- Send sample for sequencing to Cornell Biotechnology Center.
- Confirm sequence results by "blasting" or other bioinformatic measures.

Sequencing vector-subcloned PCR products

Single band is seen after electrophoretic separation of PCR reaction

- Subject total PCR sample to gel agarose gel electrophoresis. Protocol 5.6
- Extract DNA of interest from gel using mini elute gel extraction kit (Qiagen). Protocol 5.6
- Measure DNA concentration or yield by UV-spectrophotometry. Protocol 5.7
- Measure DNA purity by UV-spectrophotometry. Protocol 5.8
- Prepare PCR sequencing reaction. Protocol 5.9
- Send sample for sequencing to Cornell Biotechnology Center.
- Confirm sequence results by "blasting" or other bioinformatic measures.

Subclone PCR product into Blunt Zero vector (Invitrogen). Protocol 5.10
- Transform vectors into Top 10 chemically competent cells. Protocol 5.11
- Amplify vectors in culture and isolate vectors. Protocol 5.12
- Verify insertions by Supermix PCR and universal primers. (Invitrogen) Protocol 5.13
- Measure DNA concentration or yield by UV-spectrophotometry. Protocol 5.7
- Measure DNA purity by UV-spectrophotometry. Protocol 5.8
- Prepare PCR sequencing reaction. Protocol 5.14
- Send sample for sequencing to Cornell Biotechnology Center.
- Confirm sequence results by "blasting" or other bioinformatic measures.
General Formatting

This thesis was formatted into three distinct chapters for the purpose of aiding the reader in understanding the distinct approaches utilized in the generation of this body of this work, and for future publication considerations. Chapter 2: Immunohistochemistry, has been accepted for publication, and is included as a reprint of the original published article. General sections were included to provide a cohesive overview of the goals, materials and methods, and thought process involved in this thesis. Three appendices were included as reference sources for future lab members, and to include supplemental data for completeness.
Chapter 1: Antigen Retrieval

Optimization of Kit-like immunoreactivity within the zebrafish gastrointestinal tract through antigen retrieval.

Scott Leddon

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Brockport, New York
Abstract

Kit immunoreactivity is the most common and efficient way to visualize interstitial cells of Cajal (ICC). ICC function as regulators of gastrointestinal (GI) slow wave propagation and are essential for coordinated contraction of the GI tract. Deficiencies in ICC populations in humans have been observed in diabetic gastroparesis, irritable bowel syndrome, Hirschsprung's disease, and other motility disorders. Routine fixing procedures often mask ICC antigen recognition sites for primary antibody binding; therefore, antigen retrieval techniques are sometimes essential for consistent immunostaining. Several antigen retrieval methods were assayed to optimize conditions for paraformaldehyde-fixed paraffin-embedded zebrafish GI tissues with the anti-Kit polyclonal antibody, ab16832 (Abcam).

1. Introduction

Interstitial cells of Cajal (ICC) propagate the electrical slow wave throughout the gastrointestinal (GI) tract smooth muscle, which functions to regulate and coordinate smooth muscle contraction. ICC are necessary for coordinated motility of the GI tract, and many motility disorder in humans display lesions in ICC populations. ICC express the receptor tyrosine kinase Kit, and require Kit signaling for maintenance and survival.
Kit immunohistochemistry is the most simple and commonly accepted manner in which to visualize and analyze ICC populations.\textsuperscript{1-7} Initial attempts at Kit Immunohistochemical localization of ICC within zebrafish paraformaldehyde fixed paraffin-embedded GI tissues failed. This initial failure to visualize Kit immunohistochemistry within the zebrafish GI tract led us to believe the Kit antigen was paraformaldehyde sensitive, and would need optimized antigen retrieval conditions for consistent immunohistochemical staining.

Formalin-based fixed paraffin-embedded tissue is the most commonly used method to preserve tissue morphology by both clinicians and researchers. Tissue fixation is thought to occur primarily due to protein cross-linking. Formalin-based fixation is theorized to be a two-step reaction: addition of the aldehyde to a primary amino group forming an iminium \textsuperscript{•} intermediate, which then reacts with an electronegative carbon on an adjacent amino acid resulting in the loss of water and cross-linking of residues (figure 1).\textsuperscript{15} Cross-linking of amino acid residues can result in subtle alterations of tertiary protein structure, which is thought to be the primary cause of loss of antigen immunogenicity.
Advances in histology have yielded many techniques to restore immunogenicity of antigens in formalin-based fixed tissues; these procedures are commonly referred to as antigen retrieval or antigen unmasking. Antigen retrieval techniques aim to break amino acid cross-linking through hydrolysis and denaturation of proteins. Non-enzymatic antigen retrieval techniques aim to break or lessen the effects of amino acid cross-links induced by aldehyde based fixation. The breaking or loosing of aldehyde induced protein cross-links is accomplished primarily through common methods of protein denaturation: high temperatures, differential ionic, and altered pH conditions.

![Figure 1. Potential mechanism for formalin based fixation and resulting changes in antigenicity.](image)
Additional non-enzymatic antigen retrieval *modi operandi* include: rehydration of tissue, precipitation of soluble proteins, removal of residual traces of paraffin from tissue post deparaffinization, reversal of Schiff bases induced by aldehyde based fixatives, and disruption of salt/protein complexes that can form during tissue fixation.\textsuperscript{16-22} Enzymatic antigen retrieval, on the other hand, utilizes proteases to break limited numbers of peptide bonds, which can relive fixation induced conformational changes within the protein due to cross-linking. Additionally, protease mediated antigen retrieval may restore antigenicity by increasing tissue permeability through the digestion of extracellular and membrane bound proteins.\textsuperscript{23} Optimal retrieval techniques are antigen and fixation specific. Important variables that must be optimized include time, pH, temperature, heating method, and retrieval solutions. This paper defines optimal antigen retrieval methods for immunostaining paraformaldehyde-fixed paraffin-embedded zebrafish GI tissues with the anti-Kit polyclonal antibody, ab16832 (Abcam).

2. Materials and Methods

2.1.a Tissue

Adult wild type zebrafish (AB strain, obtained from the Zebrafish International Resource Center, ZIRC) were fixed in 4\%
paraformaldehyde (PF) in 1X PBS (Fisher Scientific; Pittsburgh PA) overnight at 4°C on a rotating platform with heads and tails removed. Gastrointestinal tracts were dissected, washed in 1X PBS, and placed in 70% ethanol. Tissues were processed with a Tissue-Tek VIP on a 12 hour protocol, and dissected into anterior, middle, and posterior segments prior to paraffin embedding. Tissues were sectioned at 4um and placed on commercially prepared charged slides (Fisher Scientific; Pittsburgh PA). Slides were baked at 56°C overnight prior to antigen retrieval.

2.2. Antigen retrieval solutions

Several solutions were tested for suitable antigen retrieval. Citraconic acid antigen retrieval solution is 0.05% citraconic anhydride in deionized water (pH 7.4). Citrate antigen retrieval solution is 10mM tri-sodium citrate dehydrate and 0.05% Tween 20 in deionized water (pH 6.0). Proteinase K antigen retrieval solution is 20µg/mL proteinase K in TE buffer

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(pH 6.0). TE buffer is 50mM tris base and 1mM EDTA in deionized water (pH 8.0). Abcam and Dako commercially available antigen retrieval solutions were diluted to manufacture recommended concentrations with deionized water; both solutions are supplied at 10X concentrations.

2.3. Antigen retrieval conditions

Tissue sections were de-paraffinized with xylene and rehydrated through an ethanol gradient, ending with deionized water. Tissue was then placed in 100°C antigen retrieval solution for 20 minutes. Tissue, still in antigen retrieval solution, was allowed to cool for 20 minutes at room temperature. Samples were then washed in two changes of phosphate buffered saline with 0.1% Triton X-100 and 0.05% Tween 20 (PBSTT) for 5 minutes each. Control tissue was placed in room temperature PBSTT instead of 100°C antigen retrieval solution. Proteinase K antigen retrieval differs from the other retrieval methods due to its proteolytic modus operandi. Post rehydration, proteinase K retrieved samples were placed in freshly prepared 37°C proteinase K antigen retrieval solution for 10 minutes; following 37°C incubation, samples were allowed to cool for 10 minutes at room temperature, and then were washed in two changes of PBSTT for 5 minutes each.
2.4. Immunohistochemistry

Tissue was blocked with 10% normal donkey serum (NDS) in PBSTT for 1 hour at room temperature, followed by incubation with Abcam 16324 1:100 (5% NDS in PBSTT) for one hour at room temperature. Tissue was washed in two changes of PBSTT for 5 minutes each prior to the application of donkey anti-rabbit IgG conjugated to Cy3 1:400 (2.5% NDS in PBSTT) for one hour at room temperature. Tissue was then washed in two changes of PBSTT for 5 minutes each. Slow Fade (Molecular Probes) was then applied before coverslipping. Fluorescence images were collected on an upright Olympus BX-51 using a Q-imaging 12-bit cooled CCD camera and Image Pro Plus software (Media Cybernetics).

2.5. Qualitative Evaluation of Antigenicity Restoration

Fluorescent images were blindly evaluated for qualitative restoration of antigenicity on the bases of immunohistochemical reactivity, nonspecific background staining, tissue integrity, and uniformity in staining (Table 2). Samples which display qualitative increases in Kit-like immunohistochemistry and nonspecific background staining levels comparable to non-retrieved samples are referred to as having an increased signal to noise ratio.
3. Results

Kit immunohistochemistry has been used in many vertebrate model systems to identify interstitial cells of Cajal in the gastrointestinal (GI) tract. Initial immunohistochemical analysis of Kit-like immunoreactivity of 4% paraformaldehyde fixed, paraffin-embedded tissue sections of adult zebrafish gastrointestinal tracts yielded negative results. However, these results were inconsistent with reverse transcriptase polymerase chain reaction performed on total RNA isolated from adult zebrafish GI tissues (Chapter 3: Molecular Biology). Further, Kit-like immunoreactivity was observed in whole-mount tissue preparations from adult zebrafish GI tract. Therefore, we used antigen retrieval techniques to test the possibility that antigen masking artifacts on paraffin embedded sectioned tissues interfered with Kit-like immunohistochemical analysis.

Five separate antigen retrieval solutions were compared, two commercially available and three commonly used. Each solution was assayed for the ability to enhance immunohistochemical staining of putative ICC, nonspecific background staining levels, tissue integrity post retrieval, and degree of uniformity in staining. The effect of proteinase K on Kit-like immunoreactivity was also determined.
Kit-like immunoreactivity is predicted to localize to the muscularis externa, the muscular outer layer of the GI tract, where ICC functionally determine the frequency and magnitude of smooth muscle contractions (see figure 2 and figure 3 for area of predicted kit-like immunoreactivity). More specifically, a high density of ICC are expected to populate the myenteric plexus region between the longitudinal and circular smooth muscle layers, similar to the mouse, guinea pig, canine, equine, and human. ICC have also been observed in high density within the deep muscular plexus, a thin band of smooth muscle adjacent to the superficial border of the circular smooth muscle, and interspersed within the smooth muscular layers.

Smooth muscle layers of the zebrafish GI tract are 2 - 8 cell layers thick, and ICC are expected to populate a 1 cell layer-network in the myenteric plexus region. The zebrafish smooth muscle layers are thin when compared to humans and other mammalians models; therefore, ICC may not be necessary to amplify and propagate the electrical slow wave across the smooth muscle layers. ICC within the circular muscle layer function to amplify the electrical slow wave and are necessary for coordinated peacemaking of GI contraction. Kit-positive cells in the zebrafish are expected to be small and the myenteric plexus layer is expected to be thin when compared to mammalian models; detection of Kit-positive cells will require high sensitivity.
The utility of each antigen retrieval solution is based on the ability of the solution to increase positive immunoreactivity, while maintaining low to moderate levels of nonspecific background staining. Optimal antigen retrieval solutions will restore positive immunostaining uniformly throughout the sample, and maintain adequate tissue integrity throughout the immunohistochemical analysis. Non-retrieved sample lacked immunostaining (figure 4), and were utilized as a base for comparison of retrieval methods. In non-retrieved samples (figure 4), the intensity of Kit-like reactivity is not distinguishable from the punctuate background staining. This lack of staining suggested possible masking effects due to fixation artifacts, and led to the evaluation of several antigen retrieval techniques for enhancement of Kit-like immunoreactivity.

Kit-like immunoreactivity is optimized by citraconic acid (figure 5), citrate (figure 6), and Dako (figure 7) solutions. The Abcam (figure 8) solution mediated retrieval of only portions of tissue sections, but the immunoreactivity areas were robust. Protease K retrieval (figure 9) was ineffective and greatly damaged the tissue.
Figure 2. 20X transverse section of the zebrafish GI tract stained with hematoxylin and eosin. The myenteric plexus is highlighted in green and the deep muscular plexus is highlighted in blue; these two plexuses are predicted by this thesis to be sites of kit-like immunoreactivity.
Figure 3. 40X transverse section of the zebrafish GI tract stained with hematoxylin and eosin. The myenteric plexus is highlighted in green and the deep muscular plexus is highlighted in blue; these two plexuses are predicted by this thesis to be sites of kit-like immunoreactivity.
Figure 4. Kit-like immunohistochemistry without antigen retrieval. Low (A, 20X) and high (B, 40X) magnification in transverse sections is indistinguishable from background staining.
Figure 5. Citraconic acid retrieval solution improves Kit-like immunohistochemistry. Kit-like immunoreactivity in transverse sections is observed in 2 distinct layers within the muscularis externa of the adult zebrafish GI tract. Low (A, 20X) and high (B, 40X) magnification fluorescent micrographs show that citraconic acid retrieval solution enhances Kit-like antigenicity, does not increase background staining, and maintains tissue morphology. Examples of Kit-like immunoreactivity within the myenteric and deep muscular plexuses are shown by the blue and red arrows respectively.
Figure 6. Citrate retrieval solution improves Kit-like immunohistochemistry. Kit-like immunoreactivity in transverse sections is observed in 2 distinct layers within the muscularis externa of the adult zebrafish CI tract. Low (A, 20X) and high (B, 40X) magnification fluorescent micrographs show that citrate retrieval solution enhances Kit-like antigenicity, increases background staining, and maintains tissue morphology. Examples of Kit-like immunoreactivity within the myenteric and deep muscular plexuses are shown by the blue and red arrows respectively.
Figure 7. DAKO commercially available retrieval solution improves Kit-like immunohistochemistry. Kit-like immunoreactivity in transverse sections is observed in 2 distinct layers within the muscularis externa of the adult zebrafish CI tract. Low (A, 20X) and high (B, 40X) magnification fluorescent micrographs show that DAKO retrieval solution enhances Kit-like antigenicity, slightly increases background staining, and maintains tissue morphology. Examples of Kit-like immunoreactivity within the myenteric and deep muscular plexuses are shown by the blue and red arrows respectively.
Figure 8. Abcam commercially available retrieval solution improves Kit-like immunohistochemistry. Kit-like immunoreactivity in transverse sections is observed in 2 poorly defined layers within the muscularis externa of the adult zebrafish CI tract. Low (A, 20X) and high (B, 40X) magnification fluorescent micrographs show that Abcam retrieval solution enhances Kit-like antigenicity, significantly increases background staining, and slightly damages tissue morphology. Examples of Kit-like immunoreactivity within the myenteric and deep muscular plexuses are shown by the blue and red arrows respectively.
Figure 9. Protease K retrieval solution does not improve Kit-like immunohistochemistry. Low (A, 20X) and high (B, 40X) magnification fluorescent micrographs show that Protease K retrieval solution does not enhance Kit-like antigenicity, significantly increases background staining, and severely damages tissue morphology.
Several non-enzymatic antigen retrieval techniques were successful at retrieving Kit-like immunoreactivity to levels above background. Citraconic acid (figure 5) retrieved Kit-like immunoreactivity with the best signal to noise ratio, maintained tissue integrity, and resulted in consistent and uniform staining. Citrate (figure 6) and Dako (figure 7) solutions maintained tissue integrity well, induced immunoreactivity. However, the Dako solution (figure 7) had a slightly better signal to noise ratio then the citrate solution (figure 6). The Abcam solution (figure 8) was able to mediate detectable Kit-like immunoreactivity; however, Abcam retrieved samples showed more tissue disruption and less uniform staining then other tested non-enzymatic retrieval solutions.

In converse to the success of several non-enzyme mediated antigen retrieval solutions, enzymatically retrieved samples utilizing protease K (figure 9), resulted in ineffective unmasking of antigen. Protease K treated samples (figure 9) displayed enhanced background staining, without enhancing Kit-like immunoreactivity; in addition, the treatment was very destructive to tissue integrity. Due to the deleterious effect on tissue integrity and lack of enhancement Kit-like signal to noise, protease K mediated enzymatic antigen retrieval is not recommended.
All of the assayed non-enzymatic methodologies of Kit-like antigen retrieval were adequate to enhance Kit-like immunoreactivity above the level of background; clearly, citraconic acid mediated retrieval (figure 5) was the most effective of all antigen retrieval techniques assayed. Dako (figure 7) and citrate (figure 6) solutions were roughly equivalent in their retrieval capabilities; the Dako solution (figure 7) resulted in slightly greater enhancement of Kit-like immunoreactivity, while maintaining slightly lower levels of non-specific background staining. The Abcam solution (figure 8) did mediated enhancement of Kit-like immunoreactivity, but often led to less uniform staining and greater tissue disruption than did citraconic acid (figure 5), Dako (figure 7), or citrate (figure 6). Overall, protease K (figure 9) treatment had a net negative effect on Kit-like immunoreactivity, and therefore is not recommended. The overall results of this comparison study are summarized in table 2.

Table 2. Effects of Various Antigen Retrieval Conditions on Kit-like Immunoreactivity

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IM: Relative estimation of immunohistochemical reactivity: — negative, + weakly positive, ++ positive, +++ strongly positive
BG: Relative estimation of non-specific background staining: — negligible, + weak, ++ moderate, +++ strong
TI: Relative estimation of tissue integrity post retrieval: — little or no damage, + slight damage, ++ moderate damage, +++ severe damage or loss of tissue
U: Relative estimation of uniformity in staining pattern: — punctuate, + partial, ++ moderately uniform, +++ uniform
4. Discussion

ICC identification most commonly relies on Kit immunohistochemistry, but the most reliable method is electron microscopy. Immunohistochemistry relies upon antibody-dependent protein identification, which gives rise to the possibility of a non-ICC cell type expressing the Kit protein; hematopoietic and mast cells in mammalian tissues are known to express Kit. The available antibodies used for this study were developed for human antigens, which raises the possibility that zebrafish Kit orthologues are not specifically detected by these reagents. Thus, anatomical location and cell morphology must be taken into account when using Kit immunoreactivity to visualize ICC. The myenteric plexus is juxtaposed between the circular and longitudinal layers of the GI smooth muscle of the tunica muscularis. ICC within the myenteric plexus of the GI tract are of most interest; myenteric plexus ICC are closely associated with enteric neurons, have bipolar or stellate processes, and are arranged in a well organized network.

The zebrafish has great potential as a model for GI dysmotility, ICC function, and drug discovery; therefore, optimization of Kit-like immunohistochemical detection within the zebrafish GI tract is desirable. Zebrafish are optically transparent as larvae, and have fully functional GI tracts by five days post fertilization. It is possible to use
immunohistochemistry to visualize GI tract cells in the whole larvae at a fully functional developmental stage, and quantify GI contractions in living fish. Larvae directly absorb small drug molecules, which makes the zebrafish attractive to drug developers for the combinatorial library screening. It has recently been shown in adult tissue and whole mount of larval and adult tissue that Kit-like immunoreactivity is present in the GI system of the zebrafish. Expression of mRNA for the kit receptors and kit ligands has been identified in the zebrafish GI tract. Antigen retrieval is necessary for identification of Kit-positive cells in transverse sections of GI tissues using the anti-Kit polyclonal antibody, ab16832 (abcam) in paraformaldehyde-fixed, paraffin embedded tissues. Citraconic acid antigen retrieval solution resulted in the most intense and uniform immunoreactivity of the assayed solutions. Additionally, citraconic acid mediated antigen retrieval did not significantly compromise tissue integrity, nor did it raise non-specific background staining above acceptable levels.
## 5. Works Cited


Chapter 2: Immunohistochemistry

Kit-like immunoreactivity within the zebrafish gastrointestinal tract.

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Kit-Like Immunoreactivity in the Zebrafish Gastrointestinal Tract Reveals Putative ICC


Gastrointestinal (GI) motility results from the coordinated actions of enteric neurons, interstitial cells of Cajal (ICC), and smooth muscle cells. The GI tract of the zebrafish has a cellular anatomy that is essentially similar to humans. Although enteric nerves and smooth muscle cells have been described, it is unknown if ICC are present in the zebrafish. Immunohistochemistry and PCR were used to determine expression for the zebrafish Kit orthologue in the zebrafish gastrointestinal tract. Cells displaying Kit-like immunoreactivity were identified in the muscular layers of the adult zebrafish gastrointestinal tract. Two layers of Kit-positive cells were identified, one with multipolar cells located between the longitudinal and circular smooth muscle layers and one with simple bipolar cells located deep in the circular muscle layer. Primers specifically designed to amplify mRNA coding for two zebrafish kit genes, kita and kitb, and two kit ligands, kitla and kitlb, amplified the expected transcript from total RNA isolated from zebrafish GI tissues. The Sparse mutant, a kita null mutant, showed reduced contraction frequency and increased size of the GI tract indicating a functional role for kita. These data establish the presence of a cellular network with Kit-like immunoreactivity in the myenteric plexus region of the zebrafish GI tract, adjacent to enteric neurons. Expression of kita and kitb, and the ligands kitla and kitlb, were verified in the adult GI tract. The anatomical arrangement of the Kit-positive cells strongly suggests that they are ICC. Developmental Dynamics 236:903-911, 2007. © 2007 Wiley-Liss, Inc.

Key words: Kit immunohistochemistry; zebrafish; interstitial cells of Cajal (ICC); gastrointestinal tract motility

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INTRODUCTION

Gastrointestinal (GI) motility is primarily mediated by complex interactions between enteric neurons, interstitial cells of Cajal (ICC), and smooth muscle cells. These cell types have been identified and characterized in many vertebrate model systems including the mouse, guinea pig, rat, dog, chick, and human (Faussone-Pellegrini and Thuneberg, 1999; Komuro, 1999; Sanders et al., 1999; Young, 1999). It is now widely understood that ICC play a vital role in regulating GI motility. ICC generate a pacing signal that drives smooth muscle, mediates neuronal input to smooth muscle, and establishes a smooth muscle membrane potential gradient across the thickness of the circular smooth muscle layer (Huizinga et al., 1995; Farrugia et al., 2003; Stroge et al., 2003; Ward et al., 2004; Sanders et al., 2006). Disturbances in ICC distribution have been correlated with GI dysmotilities in animal model systems, and in humans (He et al., 2000, 2001; Huizinga et al., 2001; Lyford et al., 2002; Sanders et al., 2006). The zebrafish is a well-established model system for studies on development, and is an important model system for human disease (Dodd et al., 2000; Goldsmith, 2004). Zebrafish larvae are transparent, which allows direct observation of organ function in the intact organism. The GI tract is functional and is apparently fully formed at 5 days post fertilization (dpf). Mus-
cular contractions can be directly observed in unperturbed intact larvae. Therefore, the functional role played by enteric neurons, ICC, and smooth muscle can be examined within an intact, living network unlike other model organisms where direct observation of GI organogenesis and GI function is not possible.

Establishing a zebrafish-based model system for GI motility requires identification of enteric neurons, ICC, and smooth muscle cells in the GI tract, as well as the characterization of function at developmental time points. Smooth muscle cells are evident at 50 hr post fertilization (hpf) and the longitudinal and circular muscle layers are well established by 96 hpf (Wallace et al., 2005a). Enteric neurons are observed by 72 hpf, completely populate the GI tract by 96 hpf, and increase in number to 120 hpf (Kelsh and Eisen, 2000; Shepherd et al., 2004). Therefore, both enteric neurons and smooth muscle cells appear well established by 5 dpf, coinciding with observations of spontaneous GI contractions and feeding (Holmberg et al., 2004). For comparison, ICC in the mouse model begin to develop at embryonic day 16, irregular and spontaneous electrical rhythmicity develops by embryonic day 18, and adult-like slow waves were recorded 9 days after birth (Ward et al., 1997; Beckett et al., 2006) ICC play a vital role in developing coordinated GI contractions that function to mix and propel the luminal contents of the gut (Sanders et al., 1997; Komuro, 1999). Deep muscular plexus ICC are typically bipolar and are oriented in parallel with circular smooth muscle, and receive excitatory and inhibitory synaptic input from the enteric nervous system (Wang et al., 1998; Wang et al., 2003; Iino et al., 2004).

Two orthologs of the mammalian c-Kit receptor tyrosine kinase have been identified in the zebrafish, kita and kitb (Parichy et al., 1999; Mellgren et al., 2005). The zebrafish Sparse mutant is characterized by a deficit in stripe melanocytes resulting from a null allele of kita (Parichy et al., 1999). Zebrafish kita plays an essential role for the migration and survival of embryonic melanocytes, but the role and/or expression of kita and kitb in the GI tract have not been reported. Because c-Kit signaling is essential for the normal development of ICC and rhythmic activity in the mouse GI tract, identification of the kita and kitb protein in the zebrafish GI tract as well as functional motility differences in the Sparse mutant would support the presence of zebrafish ICC (Ward et al., 1994; Huisinga et al., 1995; Kluppel et al., 1998). Antibodies specific for the protein label ICC within GI tissues in several species, including humans, and have been widely utilized to both determine ICC cellular morphology, and to characterize sub-populations of ICC located in distinct layers of the muscular wall of the GI tract (Ward and Sanders, 1992; Ward et al., 1994; Burns et al., 1997; Ozaki et al., 2004; Komuro, 1999). Several classes of ICC have been identified. Each class is distinguished according to cellular morphology, anatomical location, and function (Faussone-Pellegrini and Thuneberg, 1999; Komuro, 1999; Sanders et al., 1999). The myenteric plexus region, located between the circular and longitudinal muscle layers, exhibits the highest ICC density and the myenteric ICC network is continuous throughout the mammalian GI tract (Burns et al., 1997; Hirist and Edwards, 2004). A second network of ICC, termed deep muscular plexus ICC, is located between the thin innermost and thicker outer layer of circular muscle of the mammalian small intestine (Burns et al., 1997; Komuro, 1999). Deep muscular plexus ICC are typically bipolar and are oriented in parallel with circular smooth muscle, and receive excitatory and inhibitory synaptic input from the enteric nervous system (Wang et al., 1998; Wang et al., 2003; Iino et al., 2004).

Identification of ICC in the GI tract may be accomplished using Kit as a selective and specific marker (Huisinga et al., 1995; Burns et al., 1997; Kluppel et al., 1998; Faussone-Pellegrini and Thuneberg, 1999; Komuro, 1999; He et al., 2000; 2001; Lyford et al., 2002) The proto-oncogene c-kit is expressed by ICC located within the tunica muscularis of the GI tract of mice, guinea pigs, rats, dogs, and humans. Antibodies specific for this protein label ICC within GI tissues in several species, including humans, and have been widely utilized to both determine ICC cellular morphology, and to characterize sub-populations of ICC.

RESULTS

Kit expression in the zebrafish GI tract was identified using a rabbit polyclonal antibody specifically designed to recognize C terminal amino acids 961-976 of human c-Kit. A continuous and extensive network of cells displaying Kit-like immunoreactivity was observed within the muscular layers of paraformaldehyde-fixed adult zebrafish GI tract (Fig. 1). Two distinct populations of cells were observed in separate layers of whole mount tissue. One layer displayed elongated cell bodies with multiple branching processes forming a loose but regular network pattern (Fig. 1B), and a second layer was comprised of bipolar or simple bifurcating cells located deeper within the circular muscle layer (Fig. 1C). Images shown are taken from mid-intestinal segments, as classified by Wallace et al. (2005a). Substantial differences in distribution of Kit-positive cells in each segment were not observed.

Spontaneous contractions of the zebrafish GI tract begin at 4 dpf, coinciding with the development of enteric neurons, but the possibility for ICC contributing to the development of rhythmic contractions has not been explored (Kelsh and Eisen, 2000; Shepherd et al., 2004; Wallace et al., 2005b). Kit expression was identified in the GI tract of paraformaldehyde-fixed zebrafish larvae. The GI tract was carefully dissected from larvae prior to immunostaining to maximize antibody penetration of intact tissues. Single confocal sections taken midway through the digestive tube are shown for 7, 11, and 20 dpf larvae (Fig. 1D-F, respectively). Cells displaying Kit-like immunoreactivity were observed in the outer layer of the tunica muscularis, indicated by arrowheads. Kit expression appeared to increase from 7 to 11 dpf, and it was possible to identify an apparent network of Kit-positive cells at 20 dpf. Kit-like immunoreactivity was not observed prior to 7 dpf (data not shown). It was not possible to separate the mucosa from the tunica muscularis in adult or larvae GI tissues, which contributed to high background staining and prevented complete confocal stack reconstructions of the full-thickness digestive tract in larvae. The ACK2 rat mono-
clonal antibody that has been widely used to identify mouse ICC also specifically identified cellular networks in acetone-fixed adult and larvae zebrafish GI tissues (data not shown).

Two types of Kit-positive cells are observed at high magnification: one branching cell with prominent nuclei (black arrow) and thinner bipolar cells (Fig. 2A). The anatomical position of these cells was determined in transverse sections of adult zebrafish GI tissues. Hematoxylx and eosin stained transverse sections show that the outer longitudinal and inner circular smooth muscle layers are approximately 2 and 4-5 cell layers thick, respectively (Fig. 2B). Kit-like immunoreactivity was consistently observed in two distinct layers of cells in transverse sections of adult GI tissues (Fig. 2C). The outer layer of cells with Kit-like immunoreactivity appeared more dense and continuous, and a second thinner layer of Kit-positive cells was observed closer to the lumen. The inner layer was discontinuous and oriented in parallel with the circular smooth muscle cells. Both layers of Kit-positive cells were observed in anterior, mid, and posterior intestinal segments. The anatomical position of Kit-positive cells was also determined using transverse sections of zebrafish larvae. A single continuous layer of Kit-positive cells within the tunica muscularis of the GI tract was observed in 13 dpf larvae (Fig. 2D). The relative anatomical positions of Kit-positive cells, enteric neurons, and smooth muscle cells in the zebrafish GI tract was examined further for comparison with established mammalian model systems. A pan-neuronal antibody, anti HuC/D, was used to identify cell bodies, and anti-acetylated α-tubulin antibody was used to identify neural processes. Composite images resulting from dual labeling experiments with whole mounted tissues show that cells displaying Kit-like immunoreactivity are in the same area as neuronal cell processes, labeled with acetylated α-tubulin antibody (Fig. 3A). Composite images of transverse sections show Kit-positive cells and neural cell processes are located in the myenteric plexus region (Fig. 3B). Similarly, dual labeling with anti HuC/D and anti-Kit antibody shows neural cell bodies located near Kit-positive cells in whole mounted tissues, and in the myenteric plexus region in transverse sections (Fig. 3C and D). Enteric nerves were only occasionally observed to extend deep into the circular muscle layer near the thin inner layer of Kit-positive cells (data not shown). Dual labeling with anti-SM22 antibody to detect smooth muscle cells and anti-c-Kit antibody identified longitudinal and circular smooth muscle layers, and Kit-positive cells located between the layers in the whole mounted tissue (Fig. 3E). Smooth muscle cells were identified using an anti-Desmin antibody in transverse sections. Desmin is an intermediate cytoskeleton filament protein expressed by GI smooth muscles. Composite images of transverse sections dual labeled with anti-Desmin and anti-Kit antibody show one dense layer of Kit-positive cells positioned between the longitudinal and circular muscle layers and a second thin layer of Kit-positive cells near the innermost circular smooth muscle cells (arrowhead, Fig. 3F).

Expression of the zebrafish orthologs of mammalian c-Kit was verified by determining mRNA expression for the kita and kitb genes within GI tissues. Reverse transcriptase PCR was performed on cDNA prepared from total RNA isolated from adult zebrafish GI tissues. The presence of mRNA for two known orthologs of the c-Kit receptor, kita and kitb, as well as orthologues of c-Kit ligand, kitla and kitlb, were determined using specific primer sets for each gene, and for β-actin as a positive control (see Table 1). Primers were intron-spanning to rule out the possibility of genomic contamination. Products of the expected size for kita, kitb, kitla, and kitlb were amplified (Fig. 4). The identity of the bands was confirmed by sequencing. These data show for the first time the presence of mRNA encoding kita, kitb, and the ligands for these receptors, kitla and kitlb, in the zebrafish GI tract.

Development of ICC requires functional c-Kit signaling during embryogenesis in the mouse model, and also to...
maintain ICC in adults (Maeda et al., 1992; Torihashi et al., 1995; Beckett et al., 2006). We examined homozygous Sparse mutants spab 5 (ZDB-FISH-980202-47) for functional differences in GI motility and for differences in appearance of the GI tract to determine a role for the kita gene. Contraction frequency was reduced in Sparse mutants compared to wild-type 7 dpf larvae. Contraction frequency averaged $0.59 \pm 0.05$ (mean $\pm$ standard error, $n = 20$) contractions per minute in wild-type larvae, and $0.33 \pm 0.03$ (mean $\pm$ standard error, $n = 23$, $P < 0.05$) contractions per minute in Sparse mutants (Fig. 5). Inactivation of c-Kit by injection of the neutralizing antibody ACK2 in the mouse resulted in distension of the stomach, small intestine, and colon, and, therefore, the size of the GI tract in

Fig. 2. Cells with Kit-like immunoreactivity form a dense network in the myenteric plexus region, and a second network close to the circular smooth muscle-submucosal border. High magnification of Kit-positive cells reveals individual stellate-shaped cells (white) in a full thickness stack (A). Circular and longitudinal muscle layers of the adult GI tract are clearly observed in H&E stained transverse sections (B). Kit-positive cells are found in the myenteric plexus region, and a thin layer of Kit-positive cells is located near the circular smooth muscle-submucosal border (C). A single layer of Kit-positive cells was observed in the tunica muscularis of transverse sections of zebrafish larvae (white arrow) (D). Scale bars $= 20 \mu$m.

Fig. 3. Dual staining shows the relative position of Kit-positive cells and enteric neurons or smooth muscles. Cell morphology is shown in whole mounted adult tissues (left), and the position and orientation of each layer is shown in transverse sections (right). Composite images using anti-Kit and the anti-tubulin antibodies show Kit-positive cells (A, red) near neuronal cell processes (green, A). Composite images using the pan-neuronal antibody anti-Hu (green) show cells with Kit-like immunoreactivity (red) near neural cell bodies (C). Transverse sections show 2 separate layers of kit-positive cells. Kit-positive cells and neural processes (B) or neural cell bodies (D) were observed in the myenteric plexus region. Dual staining with anti-Kit and anti-SM22 or anti-Desmin antibodies show the relative positions of Kit-positive and smooth muscles. Dual staining in whole mount tissues shows outer longitudinal and inner circular smooth muscles (green, E) and cells with Kit-like immunoreactivity (red). Composite images of transverse sections clearly show the layers of longitudinal and smooth muscle separated by Kit-positive cells, and a second layer of kit-positive cells near the innermost border of circular smooth muscle. Scale bars $= 20 \mu$m.
Oligonucleotide Primer Sequences observed in consistently exhibited a distended GI tract, listinal bulb (data not shown). Playing Kit-like immunoreactivity were vae, 12,331 Cybernetics, version 5.0). The size of the GI tract in 7 dpf larger compared to 7 dpf/wild-type using Image Pro from fluorescent images and measured. The size of the GI tract in 7 dpf related compared to 7 dpf/wild-type larvae, 12,331 ± 467 μm² and 14,985 ± 483 μm², respectively (mean ± standard error, n = 20 wild-type, 12 Sparse, P ≤ 0.05). Adult Sparse zebrafish consistently exhibited a distended GI tract, which was most apparent in the intestinal bulb (data not shown). Cells displaying Kit-like immunoreactivity were observed in Sparse mutant larvae and larvea, and was first detected at 7 dpf. It is likely that expression of kita and kith occurs developmentally later than 4 dpf, or that mRNA levels in the 4-dpf GI tract occur at very low and not detectable levels by in situ hybridization techniques. Functional data show that spontaneous contractions of the GI tract that develop near 4 dpf are poorly organized (Holmberg et al., 2004). Coordinated contractions, which are better organized and contribute to propulsive motility movements, develop gradually between 5 and 7 dpf, consistent with the time course of appearance of Kit immunoreactivity (personal observation). The role of ICC on intestinal transit has been well characterized in the mouse model and mutant mice lacking ICC exhibit spontaneous but poorly organized contractions and delayed intestinal transit (Torihashi et al., 1995, 1997; Ward et al., 1996). The poorly organized contractions in early zebrafish development are, therefore, similar to the ICC-deficient mutant mouse model. The morphological data from these studies are consistent with the presence of myenteric ICC and deep muscular plexus ICC, similar to the mouse small intestine. However, no obvious differences were observed in the distribution of Kit-positive cells in anterior, mid, or posterior regions of the zebrafish GI tract, whereas in the mouse model deep muscular plexus ICC distribution differs regionally along the GI tract. The functional role of Kit-positive cells in the zebrafish GI tract, as well as the relationship between Kit-positive cells and enteric neurons, remains to be explored.

**TABLE 1. Oligonucleotide Primer Sequences**

<table>
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<tr>
<th>Target</th>
<th>Sequence</th>
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R: TCA CAG GTA CAG TCA CAT CAG TGT | 564                |
| kith (DQ072166) | F: GGG AGG AAT CAC CAT CAG AA  
R: CTC AGG TGG AAA TCCTG TT | 234                |
| kital (AY929068) | F: CACAGTGTCGTCCTAFTCCA  
R: GGTGAGAGCCACCTCAGAT | 580                |
| Kitbl (AY929069) | F: GGC TGC ATT TGA ACC TGT ATC C  
R: GTG TCT CCA CAC CCTAAA GAA TCC | 542                |
| β actin (BC067566) | F: GAT ACG GAT CCA GAC ATC AGG GTG TCA TGG  
TTG GTA  
R: GAT ACA AGC TTA TAGAC AGC TTC ACC TTC TTG ACG | 580                |

**DISCUSSION**

Results presented here show that anti-Kit antibodies identify a network of cells located between the circular and longitudinal muscle layers of the adult zebrafish GI tract and a separate, second group of cells in a region analogous to the boundary between the inner and outer circular muscle in the mouse. Expression of mRNA encoding for the two zebrafish orthologues of mammalian Kit, kita, and kith, as well as two orthologues for mammalian Kit ligand, kita and kitb, was confirmed in adult zebrafish GI tissues. The Kit-positive cells were distributed in the myenteric plexus region near enteric neurons. A reduction in contraction frequency and an increase in size of the GI tract were observed in 7 dpf Sparse mutant larvae, which exhibit a null kita allele. These data are consistent with expression of zebrafish orthologous to the mammalian c-Kit receptor tyrosine kinase, kita and kitb, in the zebrafish GI tract. Furthermore, the data strongly indicate the presence of ICC in the zebrafish GI tract, which are distributed similarly to mammalian ICC, thus establishing the zebrafish as a suitable model system for mammalian GI motility.

Expression of kita and kitb was not detected previously in zebrafish larvae at 4 dpf using in situ hybridization techniques (Parichy et al., 1999; Mellen and Johnson, 2005). Our data confirm this result. Kit-like immunoreactivity was not detected in 5-dpf larvae, and was first detected at 7 dpf. It is likely that expression of kita and kith occurs developmentally later than 4 dpf, or that mRNA levels in the 4-dpf GI tract occur at very low and not detectable levels by in situ hybridization techniques. Functional data show that spontaneous contractions of the GI tract that develop near 4 dpf are poorly organized (Holmberg et al., 2004). Coordinated contractions, which are better organized and contribute to propulsive motility movements, develop gradually between 5 and 7 dpf, consistent with the time course of appearance of Kit immunoreactivity (personal observation). The role of ICC on intestinal transit has been well characterized in the mouse model and mutant mice lacking ICC exhibit spontaneous but poorly organized contractions and delayed intestinal transit (Torihashi et al., 1995, 1997; Ward et al., 1996). The poorly organized contractions in early zebrafish development are, therefore, similar to the ICC-deficient mutant mouse model. The morphological data from these studies are consistent with the presence of myenteric ICC and deep muscular plexus ICC, similar to the mouse small intestine. However, no obvious differences were observed in the distribution of Kit-positive cells in anterior, mid, or posterior regions of the zebrafish GI tract, whereas in the mouse model deep muscular plexus ICC distribution differs regionally along the GI tract. The functional role of Kit-positive cells in the zebrafish GI tract, as well as the relationship between Kit-positive cells and enteric neurons, remains to be explored.
The physiological role of kita and kitb in the zebrafish GI tract is unknown. Parichy et al. did not observe motility defects in kita null mutants (Sparse), and homozygous adults appeared normal (Parichy et al., 1999). However, motility patterns are complex, and quantification of spontaneous contractions in zebrafish larvae is notoriously difficult. Further, erratic spontaneous contractions of GI smooth muscles do not require ICC in the mouse model. Data reported here suggest that early spontaneous rhythmic contractions observed in zebrafish larvae before 7 dpf also do not require ICC, because Kit-positive cells were not observed prior to 7 dpf. Therefore, overt GI motility deficits are not predicted for the Sparse mutant during early development and would not be observed. A role for kita in ICC development is indicated by data from Sparse mutants showing a reduced frequency of spontaneous GI contractions in 7-dpf larvae. Further, the increased surface area of the GI tract in Sparse mutants is highly similar to observations of an expanded small intestine in the W/Wv mutant mouse (Maeda et al., 1992; Torihashi et al., 1995). Complete c-KIT inactivation mutants or the absence of Kit ligand is lethal for the mouse model but homozygous Sparse mutants survive to adulthood. This raises the possibility for a different physiological function for mammalian Kit and zebrafish kita.

The recent discovery of a second Kit orthologue in the zebrafish, kitb, provides the possibility that kitb may be sufficient to support partial or complete ICC development and survival in the zebrafish GI tract (Mellgren and Johnson, 2005). Survival of homozygous kita null mutants suggests that kitb at least partially supports ICC development. Kit-like immunoreactivity was also observed in Sparse larvae and adult GI tissues, indicating that kitb contributes to ICC development and maintenance. Elucidating the specific roles for the zebrafish receptor tyrosine kinase kita and kitb during ICC development in the zebrafish, and examining whether kita and kitb are co-orthologous for human c-Kit will contribute to the understanding of ICC development during pathophysiological conditions that contribute to GI dysmotility in the clinical setting.

ICC are the primary cell type that expresses c-KIT in the GI tract but mast cells are also labeled by anti-Kit antibody. The two cell types are easily distinguished because mast cells are round and granular in appearance but typical ICC display long, slender processes and an oval body (Fausson-Pellegrini and Thuneberg, 1999; Komuro, 1999). In the present study of the zebrafish GI tract, the cellular morphology of cells displaying Kit-like immunoreactivity showed multiple branching processes, or were slender and bipolar, and round cells were never observed. Definitive confirmation of these cells as ICC may require electron microscopy to examine their ultra structure to determine if they possess features associated with mammalian ICC, such as a high density of mitochondria and intermediate filaments, an absence of thick filaments, gap junction contacts with smooth muscle cells, and surface caveolae (Komuro, 1999). Characterization of the association between enteric neurons and ICC will contribute to determining the functional role and classification of ICC in the zebrafish.

The GI tract of the zebrafish lacks a stomach, and presents as a folded tube in the adult. The anterior segment, or intestinal bulb, exhibits the largest lumen and may function as a reservoir and mixing chamber, similar to a stomach. The anterior, mid, and posterior portions roughly approximate the small and large intestine with respect to absorptive function (Wallace et al., 2005a). Direct observation of muscular contractions in intact larvae show that contractions in the anterior segment are more frequent and more random when compared to mid and posterior segments where less frequent but more ordered anterograde propagations appear. It will be interesting to determine the role for enteric neurons and ICC in these distinctly different motility patterns. Although the development of contraction frequency has been reported previously, functional studies, such as assessing transit through the GI tract, are lacking (Holmberg et al., 2004).

In summary, the results in this study show Kit-like immunoreactivity in the tunica muscularis of the zebrafish GI tract and provide functional evidence for the role of kita in GI motility. Kit-positive cells in the zebrafish appear to correspond to myenteric ICC and deep muscular plexus ICC, based on cellular morphology and distribution. Expression of mRNA encoding kita, kitb, kita, and kitlb was demonstrated in the zebrafish GI tract using PCR analysis. Mutants lacking functional kita showed a decreased contraction frequency compared to wild-type zebrafish. Taken together, these data are consistent with the presence of ICC in the zebrafish GI tract, and suggest that the zebrafish may be a suitable model system for human GI physiology. More work will be required to determine if Kit-positive cells in the zebrafish GI tract initiate...
spontaneous, rhythmic contractions, transferred electrical activity along the long axis of the GI tract, and coordinated enteric neurotransmission similar to mouse ICC.

EXPERIMENTAL PROCEDURES

Aquaculture

Wild-type and Sparse mutant zebrafish (ZFIn ID 980907 and 980267) were obtained from the Zebrafish international resource center and maintained according to standard guidelines in accordance with IACUC guidelines (Westferfer, 1993). Wild-type long-finned golds (Scientific Hatcheries, Huntington Beach, CA) were also used for some preliminary experiments and no differences in immunohistochemical staining were observed when comparing strains (data not shown). Fish were maintained at 28°C in system water comprised of deionized water containing 240 mg/L Instant Ocean salts and 75 mg/L NaHCO₃ with 20% system water change each day (pH was adjusted to ~7.2, conductivity ~450 ppm). Zebrafish were fed 3 times daily, alternating Cyclopeeze (Argent, Redmond, WA) with live brine shrimp, and maintained on a 14-hr/10-hr light/dark cycle. Crosses were performed in the morning, and embryos were maintained in embryo medium in 400 ml beakers kept in a water bath set to 28°C. Larvae were fed hatchfly encapsulation, grade 0, beginning at 7 dpf, and live brine shrimp after 11 dpf (Argent, Redmond, WA).

Immunohistochemistry

Adult and larvae zebrafish were anesthetized in system water containing MS222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co., St Louis, MO) and sacrificed for immunohistochemistry. Intact larvae and freshly dissected adult GI tissues were fixed in freshly prepared 4% paraformaldehyde in phosphate buffered saline containing 0.02% sodium azide. Larvae were carefully dissected such that the GI tract was separated from the body so that the head remained attached to the GI tract. Although time-consuming, removing the GI tract in this manner allowed direct access for antibody-antigen binding, producing consistent immunostaining. Nonspecific binding of primary antibody to tissues was minimized by incubation for at least 1 hr at 4°C in blocking solution comprised of 10% normal donkey serum (NDS, Chemicon) and phosphate buffered saline containing 0.02% sodium azide, 0.1% Triton-X-100, and 0.05% TWEEN 20 (PBS- TT). Primary antibodies were diluted in PBS-TT containing 5% normal donkey serum and were applied for 24–48 hr at 4°C on an orbital platform. After washing 4 times in PBS-TT, tissues were incubated with appropriate secondary antibody conjugated to a fluorescent marker and diluted in PBS-TT containing 2.5% normal donkey serum for 24 hr at 4°C on an orbital platform. Nonspecific immunoreactivity was assessed by immunostaining tissues or larvae in an identical manner but with the primary antibody omitted. The optimal concentration for each primary and secondary antibody was determined using serial dilutions. Antibodies were applied simultaneously during dual labeling experiments, followed by a PBS-TT wash and the simultaneous application of two appropriate secondary antibodies. Tissues were washed with PBS-TT and mounted on glass slides using Slow Fade medium (Invitrogen).

Paraffin-embedded sectioned tissues.

Adult fish were anesthetized in MS 222, decapitated posterior to the gills, tails were removed to aid fixative penetration and were immersed in freshly prepared 4% paraformaldehyde in phosphate buffered saline (Fisher) with pH adjusted between 7.3 and 7.4 for a minimum of 2 hr, but not longer than overnight. Immunostaining using the ACK2 antibody requires acetone fixation, and for these experiments larvae and tissues were fixed for 15 min in ice-cold acetone. Fixed tissues were washed 4 times in PBS containing 0.02% sodium azide. Larvae were carefully dissected such that the GI tract was separated from the body so that the head remained attached to the GI tract. Although time-consuming, removing the GI tract in this manner allowed direct access for antibody-antigen binding, producing consistent immunostaining. Nonspecific binding of primary antibody to tissues was minimized by incubation for at least 1 hr at 4°C in blocking solution comprised of 10% normal donkey serum (NDS, Chemicon) and phosphate buffered saline containing 0.02% sodium azide, 0.1% Triton-X-100, and 0.05% TWEEN 20 (PBS- TT). Primary antibodies were diluted in PBS-TT containing 5% normal donkey serum and were applied for 24–48 hr at 4°C on an orbital platform. After washing 4 times in PBS-TT, tissues were incubated with appropriate secondary antibody conjugated to a fluorescent marker and diluted in PBS-TT containing 2.5% normal donkey serum for 24 hr at 4°C on an orbital platform. Nonspecific immunoreactivity was assessed by immunostaining tissues or larvae in an identical manner but with the primary antibody omitted. The optimal concentration for each primary and secondary antibody was determined using serial dilutions. Antibodies were applied simultaneously during dual labeling experiments, followed by a PBS-TT wash and the simultaneous application of two appropriate secondary antibodies. Tissues were washed with PBS-TT and mounted on glass slides using Slow Fade medium (Invitrogen).

Fluorescence and transmitted light imaging.

Tissues were examined with conventional light and fluorescence microscopy using an Olympus BX51 microscope equipped with an Optiscan z-axis controller (Prior Scientific) and supported on an anti-vibration table.
(Technical Manufacturing Corporation, Peabody, MA). Images were captured using a Spot RT digital camera (Diagnostic Instruments) or a Retiga EXi digital camera (QImaging) using Image Pro Plus software version 5.0 (Media Cybernetics). High-resolution images were collected using an Opti grid structured light imaging system (QIOP'TIC, Rochester, NY). A 100-W mercury lamp was used for epifluorescence illumination with appropriate excitation-emission filter sets for each fluorophore. A laser-scanning confocal microscope (model LSM 510, Zeiss) was used for images in Figure 1. Images were reconstructed from confocal stacks of z-series scans as indicated.

Functional GI motility measurements.

GI motility was assayed using live larvae that were incubated in embryo medium containing blue food dye to enhance contrast of the GI tract lumen. The dye did not affect larvae survival, heart rate, or GI tract contraction frequency. Larvae were fully anesthetized and mounted laterally in 1.5% agar to permit an optimal viewing of the GI tract, and to prevent drift during filming. A drop of anesthetic was placed on the agar to keep it from drying out and to keep larvae anesthetized during filming. Spontaneous GI contractions were recorded continuously for 10 min using a Cannon Optura Xir digital video camera and converted to digital format (Pinnacle Studio ADV). Contractions were counted manually at a single position in the mid-intestine during replay of digitized video at an increased rate.

Reverse Transcriptase PCR.

Experiments were done to determine if the zebrafish Kit receptors kiti and kitb, and the Kit ligand (Steel factor) kitta and kitlb, are expressed in GI tissues. Total RNA was prepared from freshly dissected GI tissues of adult wild-type zebrafish, and from whole zebrafish (used as a positive control), using the RNeasy kit (Qiagen, Valencia, CA). First-strand synthesis was performed using random decamer primers. Gene-specific PCR was performed using Taq Pfx (Invitrogen) with 1 μl of the reaction mixture from the first-strand synthesis and primers specifically designed for each gene, and run for 35 cycles. Optimal magnesium concentration and primer annealing temperature were independently determined for each primer set. Amplification products were resolved on a 2.5% agarose gel.

ACKNOWLEDGMENTS

The authors thank Dr. David Brannigan and Mary Geiger for expert technical assistance with tissue preparation and histological procedures, Kyle Leonard for H&E staining, and Jodi Davis for zebrafish care.

REFERENCES


KIT-LIKE IMMUNOREACTIVITY IN THE ZEBRAFISH GI TRACT


Paralogs of the human genes Kit and Kit ligand are expressed within the zebrafish gastrointestinal tract.

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Brockport, New York
Abstract

The Kit receptor tyrosine kinase is expressed in the interstitial cell of Cajal (ICC), mast cells, and hematopoietic stem cells in the human gastrointestinal (GI) tract. The zebrafish expresses two Kit orthologs, kita and kitb, which play a role in melanogenesis, but expression in the GI tract has not been reported. Anti-Kit antibodies specifically identify two networks of cells located in the muscularis externa of the zebrafish GI tract, which suggests the existence of zebrafish ICC. This investigation confirms the expression of the zebrafish kit receptor genes, kita and kitb, as well as the kit ligand genes, kital and kitbl, within adult wild type zebrafish AB GI tissue. Confirmation of expression was achieved through reverse transcriptase PCR and sequencing of PCR products. These data show that kita, kitb, kital, and kitbl are expressed in zebrafish GI tissues.

1. Introduction

Kit is a tyrosine kinase receptor expressed on the cell surface of ICC within the GI tract and is necessary for the development and maintenance of ICC in humans and in mammalian model systems. Studies in mice have shown that the lack of the
Kit receptor or its ligand, steel factor, leads to lesioning of ICC populations through an unknown process; lesioning of ICC appears not the result of apoptosis (negative TUNNEL assay) (personal communication to A. Rich from SJ Gibbons).\textsuperscript{4} Humans with non-functional Kit alleles display deficiencies in ICC populations and GI motility disorders.\textsuperscript{5-7} Kit receptor expression and extracellular ligand are necessary for successful ICC cell culture.\textsuperscript{8,9} If ICC are present within the GI tract of the zebrafish, then it is likely that kit and kit ligand genes are expressed to support the development and maintenance of ICC populations. Alternatively, if the kit protein is not present within the GI tract of the zebrafish, observed Kit-like immunoreactivity would indicate a novel target in the zebrafish GI tract.

The aim of this study is to define the expression profile of the Kit receptor gene paralogs, kita and kitb, and the Kit ligand paralogs, kitla and kitlb; if the kit receptor genes are expressed then it is likely that the anti-Kit antibody is reacting with the expressed zebrafish kita and/or kitb receptor tyrosine kinase. Both zebrafish kit receptors share antigenic sequence homology with the human Kit receptor; it is expected that both zebrafish kit receptors will be visualized with anti-Kit antibodies. These experiments are a necessary step in
characterizing the zebrafish as a model system for human GI motility.

2. Materials and Methods

2.1. Rational Design

Expression of the kit receptor and ligand genes were determined by reverse transcriptase polymerase chain reaction (RT-PCR) using total RNA isolated from the GI tract of adult wild type zebrafish (AB strain, obtained from the Zebrafish International Resource Center, ZIRC). Primer sets for each gene were specifically designed to discriminate genomic DNA contamination from cDNA by amplifying over intron-exon boundaries; therefore, products of vastly different size would result from amplification of genomic DNA over cDNA. Upon confirmation of proper PCR product amplification size, products were subcloned into vectors with universal priming sites for DNA sequencing. Sequence data confirmed gene identification.

2.2. Tissues, RNA Extraction, and cDNA Synthesis

GI tissue was dissected from adult wild type zebrafish (AB strain, obtained from the Zebrafish International Resource Center, ZIRC) was immediately disrupted under liquid nitrogen
with a mortar and pestle, and homogenized using QIAshredder columns (Qiagen). Total RNA was isolated with the RNeasy Protect Mini Kit (Qiagen). Integrity of the isolated total RNA was determined by formaldehyde gel electrophoresis. First strand synthesis was accomplished using the Reverse-IT 1st strand synthesis kit (ABgene), and utilized random decamers to prime RNA.

2.3. PCR Primers

Gene specific primers were designed for zebrafish kita, kitb, kitla, and kitlb using Integrated DNA Technology’s web-based primer design tool, Primer Quest (http://www.idtdna.com/Scitools/Applications/PrimerQuest/Default.aspx/). PCR primers specifically amplify products from each gene that crossed intron-exon boundaries. This approach enhances the quality of the molecular data by preventing PCR amplification.

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from genomic contamination. Primer sets were optimized for
temperature and magnesium concentrations to produce sharp bands
of expected size with the greatest amplification possible (table
1).

2.4. Cloning

PCR products were confirmed by size for each gene using
agarose gel electrophoreses. PCR products of the expected sizes
were inserted into pCR4Blunt-TOPO vectors (Invitrogen) and
chemically transformed into One Shot TOP10 chemically competent
cells (Invitrogen). Transformed cells were allowed to grow one
hour at 37°C with rotation. Cells were then plated on LB plates
with Ampicillin and allowed to grow overnight at 37°C to select
for the presence of the pCR4Blunt-TOPO vector. Individual
colonies were cultured in LB broth overnight at 37°C with
rotation. Plasmids were isolated from each culture with PureLink
Quick Plasmid Miniprep kit (Invitrogen). Absorbance values for
isolated plasmids were taken at 260nm and 280nm to determine
purity and concentration of DNA. PCR utilizing M13 prime sites
3' and 5' to the cloning site in the pCR4Blunt-TOPO vector and
agarose gel electrophoresis were used to confirm the cloning of
the proper insert into the cloning site. Plasmid DNA was
sequenced by the Biotechnology Resource Center at Cornell
University.
3. Results

Total RNA was isolated from dissected adult zebrafish GI tissues using the RNeasy Protect Mini Kit (Qiagen). The integrity of isolated RNA was determined by formaldehyde gel electrophoresis (figure 1, lane B and F). Ribosomal RNA is highly expressed and is expected to show distinct bands at the appropriate size. Clear bands corresponding to 28s, 26s, and 18s rRNAs were observed. Total cDNA lacked strong bands correlating to rRNA (an indication of RNA contamination), and showed uniform smearing suggesting uniform first strand synthesis (figure 1, lane D).

If the Kit and Kit ligand orthologs are expressed in the zebrafish GI tract then it will be possible to PCR amplify these gene products. PCR primers specifically designed for each Kit and Kit ligand gene orthologs. Primers were designed to cross intron-exon boundaries to minimize the possibility that genomic DNA would serve as template for PCR amplification. Expression of kita, kitb, kitla, and kitlb was determined using PCR on cDNA prepared from zebrafish GI tissues (figure 2). PCR products of the expected size were observed for each primer pair. PCR products were subcloned into sequencing vectors, and sequenced using M13 forward universal primer.
Sequence data of kita (figure 3), kitb (figure 4), kitla (figure 5), and kitlb (figure 6) were identical to the published sequences. These data confirm expression of the Kit receptor orthologs kita and kitb, and the Kit ligand orthologs kitla and kitlb within the wild type adult zebrafish GI tract.
Figure 1. Electrophoretogram of zebrafish wild type AB total GI tract RNA and cDNA resolved on a 1.2% formaldehyde agarose gel. Isolated total RNA (lane B and F) shows strong bands corresponding to 28s, 26s, and 18s rRNA. RT-PCR was used to produce cDNA (lane D), which showed uniform smearing; this pattern suggest successful first strand synthesis. Lanes A and G were loaded with exACTGene 50bp mini and 100bp PCR DNA ladders, respectively (Fisher Scientific).
Figure 2. Electrophoretogram of kita, kitb, kitla, and kitlb reverse transcriptase PCR products resolved on a 2.5% agarose gel. Lane A was loaded with exACTGene 50bp mini DNA ladder (Fisher Scientific). PCR products of the predicted size for primers designed to kita (564bp), kitb (234bp), kitla (580bp), and kitlb (542bp) are shown in lanes B-E respectively. Primers designed to amplify β–actin, yielded PCR products of expected size (lane F, 562bp).
Figure 3. *Kita* reverse transcriptase PCR product sequence. Sequence data confirms GI specific expression of *kita*. Blastn search identifies *Danio rerio kita* with an expect value of 0. Sequence data aligns to 204bp-714bp of *kita* published sequence (AF153446) with bl2seq.
Figure 4. Kitb reverse transcriptase PCR product sequence. Sequence data confirms Gi specific expression of kitb. Blastn search identifies Danio rerio kitb with an expect value of 0. Sequence data aligns to 279bp- 513bp of kitb published sequence (DQ072166) with blastn.
Figure 5. Kitla reverse transcriptase PCR product sequence. Sequence data confirms Gl specific expression of kitla. Blastn search identifies Danio rerio kitla with an expect value of 0. Sequence data aligns to 53bp-634bp of kitla published sequence (AY929068) with bl2seq.
Figure 6. Kitlb reverse transcriptase PCR product sequence. Sequence data confirms Gl specific expression of kitlb. Blastn search identifies Danio rerio kitlb with an expect value of 0. Sequence data aligns to 202bp-795bp of kitlb published sequence (AY929069) with bl2seq.
4. Discussion

The data presented here show for the first time expression of mRNA encoding for the zebrafish kit receptors and kit ligands in the GI tract of adult wild type AB strain zebrafish; this is consistent with the hypothesis that coordination of GI motility requires interstitial cells of Cajal. These data contrast with published work by Parichy et al., where kit expression was not observed within the GI tract of zebrafish.\(^1\) One potential explanation for the discrepancy is that kit expression was measured 48 hours post-fertilization (personal communication to A. Rich from S. Johnson). Expression of the kit receptors within the GI tract may not be detectable until later in development; in the murine GI tract, expression of Kit occurs between embryonic day 11 and 15.\(^{10,11}\) Therefore, it is possible that kita and kitb expression occur after 2 days post fertilization, and that putative ICC develop after this developmental stage.

Sequencing of PCR products from gene specific priming confirmed the reverse transcriptase PCR based expression profile of the kit genes. Sequenced portions of genes suggest that tissue specific variations of the kit genes are not present. However, it is possible that tissue specific variants may occur in non-sequenced portions. The data suggest that kita and kitla were expressed at higher levels when compared to kitb or kitlb. The differences in expression level can be seen by ethidium
bromide staining of gels utilized to separate the PCR products by size. Further experiments are necessary to verify this observation.

The human GI tract is a complex syncytium of tissues including smooth muscle, neurons, ICC, connective, epithelial, and immune cells. This work shows expression at the mRNA level of two orthologs for the human Kit gene, kita and kitb, and two orthologs for human Kit ligand, kitla and kitlb. These results are consistent with previous reports that identify putative ICC in the zebrafish GI tract using antibodies to the human Kit protein. It is possible that mast cells located within the zebrafish tunica muscularis express kita and kitb, Kit expression by mast cells has been well documented. However, mast cells are spherical and anti-Kit antibodies specifically identified slender bipolar or stellate-shaped cells, which are morphological features that characterize ICC. Future experiments are necessary to unambiguously identify ICC in the zebrafish using electron microscopy, and to determine the physiological role for ICC in the zebrafish GI tract.
5. Works Cited


General Results and Conclusions

Intestinal cells of Cajal (ICC) within the gastrointestinal (GI) tracts of mammals, function as regulators of the electrical slow wave, which coordinates the contraction of GI smooth muscle. The coordinated contraction of GI smooth muscle propels luminal contents aoraly in an ordered manner; this coordinated movement of luminal contents aoraly is termed coordinated GI motility. To perform the function of slow wave regulators, ICC have a functionally critical anatomical position, juxtaposed between smooth muscle and enteric neurons. ICC integrate neural stimulus and distribute information to smooth muscle cells to regulate coordinated smooth muscle contractions. ICC within the myenteric plexus of the GI tract are the most important subclass of ICC to coordinated motility. Singly dispersed ICC within the muscular layers act locally to help regulate smooth muscle electrical activity, and ICC within the deep muscular plexus also have been shown to regulate GI smooth muscle contractions.

ICC require Kit receptor surface expression and Kit ligand in the extracellular space for development and maintenance. ICC are most commonly identified by Kit immunoreactivity; however, Kit is expressed by hematopoietic stem cells and mast cells. Thus, cell morphology and anatomical positioning must also be considered when evaluating Kit immunohistochemistry. ICC have
two classic morphologies, stellate shaped cells and bipolar cells with enlarged cell bodies and slender processes. Hematopoietic and mast cells generally are spherical, with mast cells having a much larger volume; mast cells may also have oblate ellipsoid morphology, especially during diapedesis and degranulation. Additionally, mast cells and hematopoietic cells are rarely found within the muscular tunic, which is the location of ICC within the GI tract. With care it is quite simple to distinguish Kit immunoreactivity cells biased on cell morphology and cell anatomy, making differential staining unnecessary.

ICC populations within the zebrafish GI tract have been investigated in this thesis to support the zebrafish as a model for GI motility. Current models for GI motility have yielded few therapeutics for GI motility disorders, thus, a new model is needed. The main advantage of the zebrafish as a model system for GI motility is the transparency of the zebrafish larvae. The zebrafish has a fully functionally GI tract by 4-5 days post fertilization (dpf), which is easily observable with low power light microscopy. It may be possible to take advantage of the optic transparency of zebrafish larvae to develop high-throughput drug screens for GI motility disorders. The presence of ICC within the zebrafish GI tract is fundamental to the utility of the zebrafish as a model for GI motility. Lesions in ICC populations (particularly ICC within the myentric plexus) are directly linked to motility disorders in humans and mice.
Validating the presence of ICC within the zebrafish GI tract is therefore imperative to the utility of the zebrafish as a model for GI motility.

This investigation has shown immunohistochemically two distinct populations of ICC within the zebrafish GI tract. A dense layer of ICC is present within the myenteric plexus region of the zebrafish GI tract; these myenteric ICC are closely associated with enteric neuron cell bodies and processes. An additional, less dense layer of ICC are present within the deep muscular plexus. The deep muscular ICC are closely associated with neural processes originating from neural cell bodies within the myenteric plexus.

The anti-Kit antibody utilized within this study, ab16832 (Abcam), was generated to target human Kit protein. It is possible that the anti-Kit antibody does not target zebrafish kit receptors, and instead binds to an unknown protein correlating to a novel cell type. It was necessary to support immunohistochemical data with expression data due to the lack of knowledge about anti-Kit antibody reactivity within the zebrafish GI tract. Since Kit and Kit ligand are essential to ICC development and maintenance in mammalian systems, expression of mRNA for the kit receptor genes and kit receptor ligand genes supports the notion that ICC are present within the zebrafish GI tract. GI specific expression of the two zebrafish kit receptor genes, kita and kitb, and the kit receptor ligand genes, kitla
and kitlb, has been determined by work presented in this thesis. Expression data was investigated with a reverse transcriptase PCR approach, and verified with sequencing of reverse transcription PCR product. GI specific expression of kita, kitb, kitla, and kitlb supports both Kit immunoreactivity within the zebrafish GI tract, and ICC presence within the GI tract of the zebrafish. For the first time in zebrafish GI specific expression of kita, kitb, kitla, and kitlb is confirmed. This thesis concludes ICC are present in two populations within the GI tract of the zebrafish, a deep muscular plexus population and a myenteric plexus population.
Appendix 1: Protocols

Scott Leddon

Department of Biological Sciences

State University of New York College at Brockport

Brockport, New York
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Appendix 1.1: Protocols

Whole Fish, Larvae, or Adult Tissues to Thin Paraffin Tissue

Section

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Department of Biological Sciences

State University of New York College at Brockport

Brockport, New York
Protocol 1.1. 25X MESAB

400mg Tricaine

97.9mL diH2O

2.1ml of 1M Tris pH 9.0

pH to 7.0-7.4
Protocol 1.2 1X MESAB

Add 1 volume of 25X MESAB to 24 volumes fish water or E3 media.
Protocol 1.3 4% Paraformaldehyde

Note of caution - always work with paraformaldehyde under the fumehood. Do not inhale powder or vapors from solution, and clean all areas to protect coworkers.

1. Add 0.4g of paraformaldehyde to 90mL of diH2O in an Erlenmeyer flask (to minimize evaporation).
2. Add three drops of 1M NaOH.
3. Heat solution at 55- 60°C until all solid has dissolved (1- 3 hours). It is important not to heat solution above 60°C and to keep solution covered with a watch glass to prevent evaporation.
4. Cool solution in an ice bath for 10 minutes.
5. Add 10mL of 10X PBS to cooled solution.
6. pH to 7.4 with 1M NaOH or 1M HCl.

Solution should be made on the day of use or frozen at -20°C until use.
Protocol 1.4 Anesthetizing Fish or Larvae

1. Place fish or larvae in 1X MESAB.

2. Wait two minutes and check somatic reflexes by touching fish with a pipette or gently agitating larvae with a blast of 1X MESAB. If the fish or larvae do not respond, they are fully anesthetized. If they respond to stimulus, give MESAB more time and check again.
Protocol 1.5 Adult Zebrafish Fixation

1. Fast fish for three days prior to fixation.
2. Head and tail may be removed to aid fixation.
3. Place fish in 4% paraformaldehyde.
4. Incubate overnight at 4°C.
5. Wash fish in two changes of diH2O for 5 minutes for whole processing; alternatively, dissect out GI tract or other tissues in PBS followed by two washes in diH2O for 5 minutes. For whole mount staining do not proceed to step 6, this step is for tissues or fish to be paraffin embedded.
6. Place fish tissue in processing cassettes and wash in 70% ethanol for 10 minutes, then place cassettes into tissue processor. If whole fish are to be paraffin embedded skip step 6 and decalcify fish.
Protocol 1.6 Larvae Zebrafish Fixation

1. Fast fish for three days prior to fixation.
2. Place fish in 4% paraformaldehyde.
3. Incubate overnight at 4°C.
4. For larvae, which will be paraffin-embedded, wash fish in two changes of diH2O for 5 minutes a wash; proceed to step 5. If larvae are to be used for whole mount staining, wash in two changes of PBS for 5 minutes a wash; proceed to immunostaining, peeling larvae if necessary, do not complete steps 5-13.
5. Heat Histogel for 3-5 minutes in 100°C water bath, or until fully liquefied.
6. Drop water bath temperature to 50-60°C, and allow Histogel temperature to equilibrate.
7. Place larvae on a clean glass slide. Remove as much liquid as possible by wicking off excess liquid with a Kimwipe.
8. Position larvae in desired orientation; paint brushes work well for this.
9. To one side of the larvae, place a drop of Histogel. Allow the Histogel to slowly flow onto the larvae; avoid disturbing the desired orientation. If orientation is disturbed, quickly reposition the larvae with a micro spatula or paint brush before Histogel sets.

10. Fully cover the larvae with additional Histogel if necessary; allow Histogel to fully set.

11. Cut square/rectangle out Histogel button; make sure to cut sectioning face at 90° to make positioning during mold casting easier.

12. Remove Histogel rectangle from the slide using a razor blade as a spatula.

13. Place larvae in processing cassette; wash in 70% ethanol for 10 minutes before placing cassette into tissue processor.
Protocol 1.7 Decalcification Protocol

Stock Ammonium Hydroxide:

70mL concentrated ammonium hydroxide

750mL of diH2O

Stock Decalcification Solution:

1. Add 82mL of stock ammonium hydroxide to 1g of EDTA (MW 292.24).

2. Add more EDTA or ammonium hydroxide stock to bring the pH of the solution to 7.2. EDTA will decrease pH, while ammonium hydroxide will increase pH. Large quantities of EDTA usually have to be added to attain pH 7.2; pH change will be slow at pH values above pH 8, but will change rapidly from pH 8- pH 7.2.

3. Bring solution volume to 100mL with diH2O.

Decalcification of Adult Zebrafish:

1. Post fixation, rinse zebrafish in PBS once for 5 minutes.

2. Next, rinse zebrafish with stock decalcification solution for 5 minutes (this can be previously used or fresh solution).
3. Place zebrafish in at least 10X volume of stock
decalcification solution. Incubate for 24-48 hours at
room temperature or 4°C with rotation. When
decalcification is complete, spinal columns can be cut with
little resistance and no brittleness.

4. Tissues are now ready for paraffin infiltration
Protocol 1.8 Tissue Processing Schedules: Zebrafish Larvae

Utilize the following tissue processing schedule for automated tissue processing. Make sure to log all usages, solutions must be rotated every 5 runs. Also, make sure to remove tissue prior to running cleaning cycle.

<table>
<thead>
<tr>
<th>Zebrafish Larvae Processing Schedule</th>
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<tbody>
<tr>
<td>Reagent</td>
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</tr>
<tr>
<td>70% ETOH</td>
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<tr>
<td>95% ETOH</td>
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<td>95% ETOH</td>
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<tr>
<td>100% ETOH</td>
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<td>100% ETOH</td>
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<tr>
<td>100% ETOH</td>
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<tr>
<td>XYLENE</td>
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<td>XYLENE</td>
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<tr>
<td>XYLENE</td>
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<td>PARAFFIN</td>
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<td>PARAFFIN</td>
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<td>PARAFFIN</td>
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</tbody>
</table>
Protocol 1.9 Tissue Processing Schedules: Whole Zebrafish and GI Tract

Utilize the following tissue processing schedule for automated tissue processing. Make sure to log all usages, solutions must be rotated every 5 runs. Also, make sure to remove tissue prior to running cleaning cycle.

<table>
<thead>
<tr>
<th>Zebrafish Adult Whole Fish and Whole GI Tract Processing Schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
</tr>
<tr>
<td>70% ETOH</td>
</tr>
<tr>
<td>95% ETOH</td>
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<td>95% ETOH</td>
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<td>PARAFFIN</td>
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</tbody>
</table>
1. Preheat embedder for about 2 hours. Make sure both reservoirs are fully melted before beginning work. The machine is programmable to began work at predestinated times, but these times need to be set for 2 hours prior to actual start times. Auto start feature must be turned off after work period, or work times being held for all future weeks.

2. All working surfaces of embedder should be sprayed lightly with paraffin release agents.

3. Molds should be prewarmed on heated surface to remove residual wax, sprayed with paraffin release agents, and then place upside down to allow residual release agents to drain prior to casting the mold.

4. Drain retort of tissue processor by pressing the start button. Machine will beep when the process is completed.

5. Remove safety bar by sliding it to the left; this will cause the machine to beep.

6. Unlatch the retort and the open lid.

7. Remove cassette from the retort cage.

8. Place cassette in the paraffin bath reservoir of the tissue embedder; allow tissue to equilibrate for 10 minutes.
9. Open the cassette and alter sample if necessary (trim, dissect, or straighten); for example, whole GI tract need to be cut into anterior, posterior, and middle portions. When altering tissue, it is very important not to loose orientation; this is very, very easy to do.

10. Trigger paraffin dispensing with the foot peddle to clear lines.

11. Collect a small amount of paraffin in the mold (about $\frac{1}{4}$ of block volume), and touch mold bottom to the cold surface, in an effort to form a thin skin on the bottom of the mold.

12. Place tissue into the mold and orient; if paraffin sets before orientation is achieved, place mold on the hot surface to melt.

13. Once tissue is in proper orientation (and paraffin is still molten- very important), place a cassette back on the top of the mold.

14. Dispense paraffin onto the cassette back, completely filling the mold and the cassette back.

15. Place the mold on the cold surface and let set for 30 minutes.

16. Remove the block from the mold very carefully. Usually, trimming the excess paraffin from the mold will release the block; avoid pulling on the cassette back to remove block from mold.
17. Store blocks in refrigerator until use.
Protocol 1.11 Sectioning Paraffin Embedded Tissue

1. Cut the block face with a new razor blade as evenly as possible to expose tissue.

2. Trim block face to minimize paraffin surrounding tissue, yet keeping square shape. It is often desirable to leave an untrimmed face on the edge of the block that will strike the knife first on the down stroke; this seems to facilitate better ribboning.

3. Rehydrate block in diH2O ice bath for 30 minutes.

4. Set up room temperature diH2O bath and 56-58°C diH2O bath.

5. Clean sectioning knife with xylene and allow to completely dry.

6. Lock the advancing arm in place, and place the block into the block holder.

7. Position knife in knife holder. Make sure the cutting face points up and is as level as possible; also align the knife so that the bevel is slightly above the knife holder's face. Clamp knife into place.

8. Adjust knife angle to about 5°.

9. Adjust the block face to make it parallel with the knife. Also, adjust the block face to make the top and bottom equal distance from the knife during the cutting stroke.
10. Move the knife holder as close as possible to the block without touching it; lock knife holder in place.

11. Unlock the block advance arm, and rotate it clockwise to begin cutting.

12. Advance arm until cutting of the block begins. Observe which parts of the block are being cut, and for example adjust as follows: if the bottom of the block is cutting, but the top is not, adjust the block so that the top of the block come slightly forward and the bottom of the block comes slightly back. Make sure to lock the knife advancement arm before adjusting the block.

13. Once uniform sections are being cut, if proper ribboning is not achieved, adjust the knife angle.

14. Once uniform cutting and ribboning are achieved transfer sections with paint brushes to the surface of a room temperature water bath; sections should spread out and uncurl. It is sometimes necessary to unroll the sections with paint brushes.

15. Collect sections on a slide by inserting the slide into the room temperature water bath at $45^\circ$; contacting the edge of the section with the adhesive treated surface; using a brush or another slide, if necessary, to coax sections into contacting the slides in desired orientation; and finally, slowly withdraw the slide from the water bath at $45^\circ$. 

16. Inserting the slide with sections into a 56- 58°C diH2O bath at 45°, make sure to retain contact between the slide and the very last position of section; sections will shrink slightly and fully unwrinkled.

17. Slowly withdraw the slide from the bath at 45° to avoid trapping water under the paraffin sections.

18. Place the slide in a drying rack at 90- 45° to dry for 1 hour.

19. Touch end of the slide to an absorptive surface to wick off excess water.

20. Place the slide onto a slide dryer or hot plate for 1 hour to overnight at 56- 58°C.

21. Store slides at -20°C wrapped in parafilm

22. Also see attached video entitled tissue sectioning.
Appendix 1.2: Protocols

Immunohistochemistry

Scott Leddon

Department of Biological Sciences

State University of New York College at Brockport

Brockport, New York
Protocol 2.1 PBS 0.02% Sodium Azide

100mL of 10X PBS

900mL of type 1 H2O

0.2g sodium azide

pH to 7.0 - 7.4
Add 100μL of Triton X-100 and 50μL of Tween 20 to 99.85mL of PBS 0.02% sodium azide, and gently mix (do not shake) until Triton is dissolved.
Protocol 2.3 0.1% 0.05% PBSTT 10% NDS

- This is blocking solution.

Add 1mL of NDS (or normal serum of the secondary Ab host) to 9mL of 0.1% 0.05% PBSTT, mix gently (do not shake).
Protocol 2.4 0.1% 0.05% PBSTT 5% NDS

- This is used for Ab 1° dilution.

Add 0.5mL of NDS (or normal serum of the secondary Ab host) to 9.5mL of 0.1% 0.05% PBSTT, and gently mix (do not shake).
Protocol 2.5 0.1% 0.05% PBSTT 2.5% NDS

- This is used for Ab 2° dilution.

Add 0.25mL of NDS (or normal serum of the secondary Ab host) to 9.75mL of 0.1% 0.05% PBSTT, and gently mix (do not shake).
Protocol 2.6 1° Ab Solution

Dilute Ab 100-800X with 0.1% 0.05% PBST 0.5% NDS.

- Dilution factors vary between Ab.
- When dual staining make sure 1° Ab are raised in different organisms, and make dilutions one-half as dilute as the final working concentration; this allows the combination of two equal volumes of 1° Abs to achieve proper final dilutions.
Protocol 2.7 2° Ab Solution

Dilute Ab 100-800X with 0.1% 0.05% PBSTT 0.25% NDS.

- Dilution factor vary between Ab.
- When dual staining make sure 2° Ab do not have the same conjugate, and that the conjugates excitation and emission (most important) wavelengths do not overlap. Make dilutions one-half as dilute as the final working concentration; this allows the combination of two equal volumes of 2° Abs to achieve proper final dilutions.
Protocol 2.8 Citraconic Acid Antigen Retrieval Solution

400μL of citraconic anhydride

1000mL of deionized water

pH to 7.4 with 10M NaOH
Protocol 2.9 Citrate Antigen Retrieval Solution

2.94g of trisodium citrate dehydrate

1000mL of deionized water

500μL of Tween 20 (gently mix to dissolve Tween 20)

pH to 6.0 with 10M HCl
Protocol 2.10 Tris EDTA Buffer (50mM Tris, 1mM EDTA, pH 8.0)

6.10g of Tris base
0.37g EDTA
1000mL of deionized water
pH to 8.0 with 10M HCl
Protocol 2.11 Proteinase K Antigen Retrieval Solution

0.2g of proteinase K

100mL of Tris EDTA buffer

Final concentration of 20µg/mL
Protocol 2.12 Abcam Retrieval Solution

Dilute 10X commercially available stock to working concentrations by adding 1 part 10X stock to 9 parts deionized water.
Protocol 2.13 Dako Antigen Retrieval Solution

Dilute 10X commercially available stock to working concentrations by adding 1 part 10X stock to 9 parts deionized water.
1. Set up the following stations in respective flow: xylene, xylene, 100% EtOH, 100% EtOH, 95% EtOH, 95% EtOH, PBS.

- The first xylene, 100% EtOH, and 95% EtOH should be rotated out every five processing sessions or when precipitate forms.
- Only dispose of xylene in a xylene waste receptacle. EtOH that has been contaminated with paraffin or xylene should be only disposed of in a mixed organics EtOH receptacle.

2. Place baked slides in slide carriage, and pass through above stages. The slides should be in each xylene stage for 5 minutes, each EtOH stage for 3 minutes, and PBS for 20-30 minutes.

- Between stations, slides should be tapped off to rid the slide of excess liquid; do not allow tissues to dry.
- If when placing slides into a new station, the station becomes cloudy- immediately change the station, your slides should be salvageable.
Protocol 2.15 Antigen Retrieval

Use antigen retrieval only when necessary. Different tissue preparations, antigens, and fixation times all can affect antigen retrieval outcome. A battery of solutions should be tested on samples that have previously not been optimized. Temperature, incubation time, pH, heating methods, and length of retrieval all influence success; it may be necessary to alter these conditions to ensure success.

Use the following procedure when using non-enzymatic antigen retrieval solutions:

1. Heat solution to 100°C in a circulating water bath.
2. Place slide or samples in solution.
3. Incubate for 20 minutes.
4. Remove antigen retrieval solution (still containing samples) from water bath, and allow cooling at room temperature for 20 minutes.
5. Wash 2X in 0.1% 0.05% PBSTT for 5 minutes a wash.
Use the following procedure when using enzymatic antigen retrieval solutions:

1. Heat solution to 37°C (or to temperature optimal for enzymatic reaction) in a circulating water bath.
2. Place slide or samples in solution.
3. Incubate for 10 minutes.
4. Remove antigen retrieval solution (still containing samples) from water bath, and allow cooling at room temperature for 10 minutes.
5. Wash 2X in 0.1% 0.05% PBSTT for 5 minutes a wash.
Protocol 2.16 Quick Immunostaining

The following protocol is for sectioned tissue on slides. If whole mounts are being stained, simply use scintillation or micro-well plates for solution changes, and begin at step 2. Place samples in well slides after step 6. Rotation may also be used for whole mount incubations and washes.

1. Dry area around tissue with a vacuum, and then use a pap pen to outline the tissue on the slides (pap pens will not work properly if used on a wet surface). Remember to work fast to avoid drying out the tissue.

2. Gently pipette blocking solution (0.1% 0.05% PBSTT 10% NDS) onto the tissue section, using enough to cover tissue. Incubate at room temperature for 1 hour (cover with a Petri dish to avoid evaporation, and check slides periodically to make sure the blocking solution does not evaporate, add more if necessary).
3. Gently vacuum off blocking solution, and gently pipette enough $1^\circ$ Ab to fully cover tissue. Incubate at room temperature for 1 hour (cover with a Petri dish to avoid evaporation—also check slides periodically to make sure the $1^\circ$ Ab does not evaporate, add more if necessary).

4. Gently vacuum off $1^\circ$ Ab. Gently pipette 0.1% 0.05% PBST onto the tissue section, using enough to cover tissue. Incubate for 5 minutes, and then gently vacuum off 0.1% 0.05% PBST. Repeat this step for a total of three washes.

5. Gently vacuum off 0.1% 0.05% PBST, and gently pipette enough $2^\circ$ Ab to fully cover tissue. Incubate at room temperature for 1 hour (cover with a Petri dish to avoid evaporation, and check slides periodically to make sure the $2^\circ$ Ab does not evaporate, add more if necessary).
6. Gently vacuum off 2° Ab. Gently pipette 0.1% 0.05% PBSTT onto the tissue section, using enough to cover tissue. Incubate for 5 minutes, and then gently vacuum off 0.1% 0.05% PBSTT. Repeat this step for a total of three washes.

7. Apply one small drop of Slow Fade compound A (Invitrogen) to the tissue sample and coverslip. The slides are now ready for observation.
Protocol 2.17 Long Immunostaining

- The following protocol is for sectioned tissue on slides. If whole mounts are being stained, simply use scintillation or micro-well plates for solution changes, and begin at step 2. Place samples in well slides after step 6. Rotation may also be used for whole mount incubations and washes.

1. Dry area around tissue with a vacuum, and then use a pap pen to outline the tissue on the slides (pap pens will not work properly if used on a wet surface). Remember to work fast to avoid drying out the tissue.

2. Gently pipette blocking solution (0.1% 0.05% PBSTT 10% NDS) onto the tissue section, using enough to cover tissue. Incubate at room temperature for 1 hour (cover with a Petri dish to avoid evaporation, and check slides periodically to make sure the blocking solution does not evaporate, add more if necessary).
3. Gently vacuum off blocking solution, and gently pipette enough 1° Ab to fully cover tissue. Incubate at 4°C for 8 hours or overnight (cover with a Petri dish to avoid evaporation).

4. Gently vacuum off 1° Ab. Gently pipette 0.1% 0.05% PBSTT onto the tissue section, using enough to cover tissue. Incubate for 5 minutes, and then gently vacuum off 0.1% 0.05% PBSTT. Repeat this step for a total of three washes.

5. Gently vacuum off 0.1% 0.05% PBSTT, and gently pipette enough 2° Ab to fully cover tissue. Incubate at 4°C for 8 hours or overnight (cover with a Petri dish to avoid evaporation).

6. Gently vacuum off 2° Ab. Gently pipette 0.1% 0.05% PBSTT onto the tissue section, using enough to cover tissue. Incubate for 5 minutes, and then gently vacuum off 0.1% 0.05% PBSTT. Repeat this step for a total of three washes.
7. Apply one small drop of Slow Fade compound A (Invitrogen) to the tissue sample and coverslip. The slides are now ready for observation.
Appendix 1.3: Protocols

RNA Isolation to cDNA

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Department of Biological Sciences

State University of New York College at Brockport

Brockport, New York
Protocol 3.1 DEPC Treated H2O (0.1% DEPC)

1mL DEPC

999mL diH2O

1. Shake vigorously until DEPC is fully dissolved.
2. Incubate 12 hours at 37°C.
3. Autoclave or heat to 100°C for 15 minutes.
Protocol 3.2 10X Formaldehyde Gel Buffer

200mM 3-([N-morpholino]propanesulfonic acid (MOPS) (free acid)- MW 202.26

50mM sodium acetate- MW 136.08

10mM EDTA- MW 292.24

pH to 7.0 with 1 M NaOH
Protocol 3.3 1X Formaldehyde Gel Running Buffer

100mL 10X formaldehyde gel buffer

20mL 37% (12.3 M) formaldehyde

880mL RNase-free H2O (DEPC treated)
Protocol 3.4 5X RNA Loading Buffer

16μL saturated bromophenol blue solution

80μL 500mM EDTA, pH 8.0- MW 292.24

720μL 37% (12.3 M) formaldehyde

2000μL 100% glycerol

3084μL formamide

4000μL 10X formaldehyde gel buffer

100μL RNase-free H2O (DEPC treated)
Protocol 3.5 1.2% Formaldehyde Gel (50mL)

0.6g agarose

5mL of 10X formaldehyde gel buffer

45mL of RNase-free H2O (DEPC treated)

1. Add the above to an 250mL Erlenmeyer flask (to minimize evaporation).

2. Heat in the microwave until agarose is fully dissolved, do not allow to over boil.

3. Cool solution to 65°C, and add under a fumehood 0.5μL of 10mg/mL ethidium bromide and 900μL of 37% (12.3M) formaldehyde.

4. Mix well, pour into mold, and allow to set for 30 minutes at room temperature.

5. Remove gel from mold and place in electrophoresis containing 1X formaldehyde gel running buffer.

6. Allow gel to equilibrate with buffer for 30 minutes prior to loading gel.
Protocol 3.6 RNA Isolation RNaseasy Mini Kit

1. 10mg-30mg of tissue as starting material (excess tissue can cause the column to clog or supersede lysis buffer capacity, resulting in inefficient RNA isolation). Wear gloves and treat all surfaces including your table, gloves, and dissection tools with RNAaway to degrade exogenous RNAases.

2. Stabilize RNA with RNAlater for best results prior to total mRNA isolation.

3. Excise fresh tissue and cut into sections of 0.5cm or less (one dimension to insure penetration). Immediately place tissue in the appropriate volume of RNAlater; 10μl per 1mg of tissue or more is appropriate, tissue needs to be fully submerged.

4. β-mercaptoethanol (bad stuff- gloves and hood) must be added to Buffer RLT before use. Add 10μl of β- mercaptoethanol per 1ml of Buffer RLT (stable for 1 month). Buffer RPE needs to be diluted with 100% EtOH before first use (add 4 volumes of EtOH before use).
5. Remove tissue from RNAlater with forceps. Do not use more than 30 mg. Place in a homogenizing vessel with liquid nitrogen. Grind thoroughly and allow liquid nitrogen to evaporate, but do not let tissue thaw. Add 350μl (under 20mg of tissue) to 600μl (20mg - 30mg of tissue) of Buffer RLT. Use additional Buffer RLT for hard to lyse tissues.

6. Pass ground tissue through an RNase free syringe with a 20-gauge needle at least 5X.

7. Centrifuge the tissue lysate for 3 minutes at max speed in a microcentrifuge. Transfer the supernate to a new tube. In some preps the pellet may be invisible, do not transfer. Use only the supernate (lysate) in the remaining steps.

8. Add one volume of 70% EtOH to lysate and mix immediately by pipetting, move to the next step.

9. Add up to 700μl of the sample (including any precipitate) to a spin column placed in a 2 ml collection tube. Close and centrifuge for 15 s at less than 8000 g (10000 rpm). Discard flow through. If you have more sample, repeat.

10. Add 700μl Buffer RW1 to the RNeasy column. Close and centrifuge for 15 s at less than 8000 g (10000 rpm). Discard flow through.
11. Transfer the column to a new collection tube. Add 500μl of Buffer RPE to column, close, and centrifuge for 15s at less than 8000g (10000rpm). Discard flow through. Before using RPE for the first time it must be diluted see above

12. Add another 500μl of Buffer RPE to column, close, and centrifuge for 2 minutes at less than 8000g (10000rpm). Discard flow through.

13. Place column in a fresh collection tube, and centrifuge at max speed for 1 minute.

14. To elute, place column in a new 1.5ml collection tube, and add 30-50μl of RNase-free water onto the filter. Close and centrifuge for 1 minute at less than 8000g (10000 rpm).

15. Analyze RNA integrity by formaldehyde gel electrophoresis.

16. Analyze RNA purity through \( \text{Abs}_{260}/\text{Abs}_{280} \).

17. Analyze RNA concentration through \( \text{Abs}_{260} \).
Protocol 3.7 1.2% Formaldehyde Gel Electrophoresis

1. Add 3μL of 5X RNA loading buffer to 12μL of RNA sample.
2. Incubate at 65°C for 3-5 minutes.
3. Chill on ice for 1 minute.
4. Load sample into equilibrated formaldehyde gel.
5. Run gel at 100mV for 2 minutes.
6. Lower voltage to 70mV until desired separation is archived.
Protocol 3.8 RNA Concentration

1. Utilize 10mM Tris·Cl pH 7.5 to zero spectrophotometer.
2. Dilute RNA 1:50 to 1:1000 in 10mM Tris·Cl pH 7.5, for absorbance values to be accurate the absorbance should be between 1.0 - 0.15.
3. Measure \( A_{260} \).
4. \[ [\text{RNA}] \mu g/mL = 40 \times A_{260} \times \text{dilution factor} \ (1:100 \equiv 100) \times \text{light path (cm)} \]
5. RNA yield \( \mu g = [\text{RNA}] \mu g/mL \times \text{volume sample mL} \).
Protocol 3.9 RNA Purity ($A_{260}/A_{280}$)

1. Utilize 10mM Tris·Cl pH 7.5 to zero spectrophotometer.
2. Dilute RNA 1:100 to 1:1000 in 10mM Tris·Cl pH 7.5, for absorbance values to be accurate the absorbance should be between 1.0 - 0.15.
3. Measure $A_{260}$ and $A_{280}$.
4. Pure RNA has a $A_{260}/A_{280}$ ratio ranging from 1.9 - 2.3.

1a. Mix the following in a thin walled PCR tube (Reaction Tube):

- 3μL of total RNA (300ng)
- 1μL of random decamers (purple cap 400ng/μL)
- 8μL RNase free water

1b. Mix the following in a thin walled PCR tube (Control Tube):

- 1μL of MS2 positive control RNA
- 1μL of random decamers (purple cap 400ng/μL)
- 10μL RNase free water

2. Heat to 70°C for 5 minutes, then place on ice.
3. Add the following to each PCR tube:

- 4μl 5X first strand synthesis buffer (red cap)
- 2μl dNTP mix, 5mM each (green cap)
- 1μl Reverse-iT RTase blend (clear cap)
- 1μl DDT 100mM (yellow cap)

4. Incubate tube at 47°C for 50 minutes.

5. Inactivate Reverse-iT RTase blend by incubating tube at 75°C for 10 minutes.

6. Store at -20°C.
Control (MS2) PCR for Reverse Transcription Reaction

1. Mix the following in a PCR tube:

- 2.50μL 10X buffer
- 0.75μL 10mM DNTP
- 0.50μL template
- 0.20μL PFX
- 19.05μL DNase free water
- 0.50μL 50mM MgSO₄
- 0.75μL 20p mole/μL forward primer
- 0.75μL 20p mole/μL reverse primer

**TOTAL VOLUME** 25μL
2. Use the following PCR program:

   Initial denaturing 2 minutes at 94°C

   30 cycles of the following:

   Denaturing 20 seconds at 94°C

   Annealing 30 seconds at 60°C

   Extension 40 seconds at 72°C

   Final Extension 5 minutes at 72°C

3. Analyze product via agarose gel electrophoresis. A single product of 462bp should be amplified.
Appendix 1.4: Protocols

PCR

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Brockport, New York
Protocol 4.1 50X TAE

242g Tris base MW 121.1

57.1mL glacial acetic acid

18.6g EDTA (disodium salt) MW 372.24

1. Add the above to -900mL diH2O.
2. pH to 8.3 with Tris base or glacial acetic acid.
3. Volume to 1L with diH2O.
Protocol 4.2 1X TAE

1. Add 20mL of 50X TAE to 980mL of diH2O.
2. pH to 8.3 with Tris base or glacial acetic acid.
Protocol 4.3 2.5% Agarose Gel (50mL)

1.25g agarose

50mL TAE, pH 8.3

1. Add the above to an 250mL Erlenmeyer flask (to minimize evaporation).

2. Heat in the microwave until agarose is fully dissolved, do not allow to over boil.

3. Cool solution to 65°C, and under a fumehood add 5μL of 10mg/mL ethidium bromide.

4. Mix well, pour into mold, and allow to set for 30 minutes at room temperature.

5. Remove gel from mold, and place in electrophoresis chamber containing 1X TAE.
Protocol 4.4 PCR Primer Dilutions

Final concentration [DNA] = 20pmole/μL

\[
\text{dilution of DNase free H2O } \mu\text{L} = \frac{\text{pmole} \cdot \mu\text{L}}{20 \text{ pmole}}
\]
Protocol 4.5 PCR Primer Temperature Optimization

**PFX PCR reaction mix (values given per reaction)**

- 2.50μL 10X buffer
- 0.75μL 10mM DNTP
- 0.50μL template
- 0.20μL PFX
- 19.05μL DNase free water
- 0.50μL 50mM MgSO₄
- 0.75μL 20pmole/μL forward primer
- 0.75μL 20pmole/μL reverse primer

**TOTAL VOLUME 25μL**

1. Add all above components, except primers, and mix well. 6 reactions per primer set will be needed.

2. Split the reaction into the number of primer sets being assessed (23.5μL per reaction or 141μL per primer set).
3. Add 0.75μL of forward and reverse primer per reaction (4.5μL each per primer set) to the appropriate tubes and mix well.

4. Split the reaction into 25μL volumes into thin walled PCR tubes.

5. Run PCR program on temperature gradient, 48°C - 63°C.

   Initial denaturing 2 minutes at 94°C

   35 cycles of the following:

   Denaturation 20 seconds at 94°C

   Annealing 30 seconds at 48- 63°C

   Extension 40 seconds at 68°C

   Final extension 5 minutes at 68°C

6. Analyze PCR products by agarose gel electrophoresis.
Supermix PCR reaction mix (values given per reaction)

23.00µL Supermix

0.50µL template

0.75µL 20pmole/µL forward primer

0.75µL 20pmole/µL reverse primer

**TOTAL VOLUME** 25µL

1. Add all above components, except primers, and mix well. 6 reactions per primer set will be needed.

2. Split the reaction into the number of primer sets being assessed (23.5µL per reaction or 141µL per primer set).

3. Add 0.75µL of forward and reverse primer per reaction (4.5µL each per primer set) to the appropriate tubes and mix well.

4. Split the reaction into 25µL volumes into thin walled PCR tubes.
5. Run PCR program on temperature gradient, 48°C- 63°C.

Initial denaturing 2 minutes at 94°C

35 cycles of the following:

Denaturing 20 seconds at 94°C

Annealing 30 seconds at 48- 63°C

Extension 40 seconds at 72°C

Final extension 5 minutes at 72°C

6. Analyze PCR products by agarose gel electrophoresis.
Protocol 4.6 2.5% Agarose Gel Electrophoresis

1. Add 5μL of 6X DNA loading buffer to 25μL of PCR reaction.
2. Mix well.
3. Load sample into agarose gel.
4. Run gel at 100mV for 2 minutes.
5. Lower voltage to 70mV until desired separation is archived.
Protocol 4.7 PCR Primer Mg\(^{2+}\) Concentration Optimization

It is important to note that SuperMix PCR cannot be optimized for magnesium concentration because magnesium is part of the supermix.

Prepare the following solutions:

4.166mM Mg\(^{2+}\)

8.32μL 50mM MgSO\(_4\)

91.68μL DNase free H\(_2\)O

8.333mM Mg\(^{2+}\)

16.66μL 50mM MgSO\(_4\)

83.33μL DNase free H\(_2\)O

16.666mM Mg\(^{2+}\)

33.33μL 50mM MgSO\(_4\)

66.66μL DNase free H\(_2\)O
PFX PCR reaction mix (values given per reaction)

2.50μL 10X buffer

0.75μL 10mM DNTP

0.50μL template

0.20μL PFX

16.55μL DNase free water

3.00μL Mg²⁺ solution

0.75μL forward primer

0.75μL reverse primer

**TOTAL VOLUME** 25μL

1. Add all above components, except primers and Mg²⁺ solution, for total number of reactions for all primer sets and Mg²⁺ concentrations (3 reactions per primer set) to be assayed and mix well.

2. Split the reaction into three equal volumes, label the tubes 0.5, 1.0, and 2.0 (20.5μL X number of total reactions)/3 = division volume.
3. Add 3μL of 4.166mM Mg\(^{2+}\) per reaction to tube 0.5 (if 9 total reactions were set up, each of the split volumes should have 3 reaction volumes, so to 0.5 tube add 9μL of 4.166mM Mg\(^{2+}\)) and mix well.

4. Repeat step 3 for tube 1.0 and 2.0 utilizing 8.333mM Mg\(^{2+}\) and 16.666mM Mg\(^{2+}\), respectively.

5. Split each tube equally between the number of primer sets to be assayed. The volume per reaction of the tubes should be 23.5μL.

6. Add proper forward and reverse primer volumes to each reaction tube (0.75μL of forward and reverse primer per reaction) and mix well.

7. Split the reaction into 25μL volumes into thin walled PCR tubes.

8. Run PCR program on optimized temperature

   Initial denaturing 2 minutes at 94°C

   35 cycles of the following:

   Denaturing 20 seconds at 94°C

   Annealing 30 seconds at 48–63°C

   Extension 40 seconds at 68°C

   Final extension 5 minutes at 68°C

9. Analyze PCR products by agarose gel electrophoresis.
Protocol 4.8 General PFX PCR for Reverse Transcription

**Reaction**

1. Mix the following in a PCR tube:

   - 2.50μL 10X buffer
   - 0.75μL 10mM DNTP
   - 0.50μL template
   - 0.20μL PFX DNA polymerase
   - 19.05μL DNase free water
   - 0.25μL 50mM MgSO₄ (0.5mM final)
   - 0.75μL 20pmole/μL forward primer
   - 0.75μL 20pmole/μL reverse primer

**TOTAL VOLUME 25μL**
2. Use the following PCR program:

Initial denaturing 2 minutes at 94°C

35 cycles of the following:

Denaturing 20 seconds at 94°C

Annealing 30 seconds at 48–63°C

Extension 40 seconds at 68°C

Final extension 5 minutes at 68°C

4. Analyze product via agarose gel electrophoresis.

5. Store reactions at -20°C until use.
1. Mix the following in a PCR tube:

23.00μL Supermix

0.50μL template

0.75μL 20pMole/μL forward primer

0.75μL 20pMole/μL reverse primer

**TOTAL VOLUME 25μL**
2. Use the following PCR program:

   Initial denaturing 2 minutes at 94°C

35 cycles of the following:

   Denaturing 20 seconds at 94°C

   Annealing 30 seconds at 48–63°C

   Extension 40 seconds at 72°C

   Final extension 5 minutes at 72°C

6. Analyze product via agarose gel electrophoresis.

7. Store reactions at -20 °C until use.
Appendix 1.5: Protocols

DNA Sequencing

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Brockport, New York
Protocol 5.1 LB (Luria-Bertani) Ampicillin Media

10g tryptone

5g yeast extract

10g NaCl

950mL deionized water

1. Mix well and pH to 7.0 with 1.0M NaOH.
2. Bring final volume to 1L with deionized water.
3. Autoclave at 15psi for 20 minutes on liquid cycle.
4. Allow to cool to about 55°C (this is when the solution can be held comfortably in hand).
5. Add 0.1g of ampicillin (final concentration 100µg/mL).
6. Store at 4°C in the dark.
Protocol 5.2 LB (Luria-Bertani) Kanamycin Media

10g tryptone

5g yeast extract

10g NaCl

950mL deionized water

1. Mix well and pH to 7.0 with 1.0M NaOH.
2. Bring final volume to 1L with deionized water.
3. Autoclave at 15psi for 20 minutes on liquid cycle.
4. Allow to cool to about 55°C (this is when the solution can be held comfortably in hand).
5. Add 0.05g of kanamycin (final concentration 50μg/mL).
6. Store at 4°C in the dark.
 Protocol 5.3 LB (Luria-Bertani) Ampicillin Plates

10g tryptone

5g yeast extract

10g NaCl

950mL deionized water

1. Mix well and pH to 7.0 with 1.0M NaOH.
2. Bring final volume to 1L with deionized water.
3. Add 15g of agar.
4. Autoclave at 15psi for 20 minutes on liquid cycle.
5. Allow to cool to about 55°C (this is when the solution can be held comfortably in hand).
6. Add 0.1g of ampicillin (final concentration 100μg/mL).
7. Mix and then pour plates.
8. Let plates harden.
9. Store inverted at 4°C in the dark.
Protocol 5.4 LB (Luria-Bertani) Kanamycin Plates

10g tryptone

5g yeast extract

10g NaCl

950mL deionized water

1. Mix well and pH to 7.0 with 1.0M NaOH.

2. Bring final volume to 1L with deionized water.

3. Add 15g of agar.

4. Autoclave at 15psi for 20 minutes on liquid cycle.

5. Allow to cool to about 55°C (this is when the solution can be held comfortably in hand).

6. Add 0.05g of kanamycin (final concentration 50μg/mL).

7. Mix and then pour plates.

8. Let plates harden.

9. Store inverted at 4°C in the dark.
Protocol 5.5 PCR Reaction Purification

1. Add 5X volume of Buffer PB to PCR reaction (5μl of buffer to 1μl of PCR reaction).

2. Add sample to MinElute column (make sure column is in a collection tube). Maximum volume of MinElute column is 800μL, if samples exceed 800μL, repeat step 2 and 3 until all sample has been applied to column.

3. Close MinElute column, centrifuge for 1 minute at 10,000 X g, and discard flow through. Reuse collection tube for step 4.

4. Add 750μL of Buffer PE to the MinElute column, let stand 2-5 minutes

5. Close MinElute column, centrifuge for 1 minute at 10,000 X g, and discard flow through. Reuse collection tube for step 6.

6. Centrifuge for 1 minute at 10,000 X g, and discard flow through. Discard collection tube and place MinElute column in a new collection tube.

7. Add 10μL of Buffer EB (10mM Tris·Cl, pH 8.5) or RNase Free H2O to the membrane of the MinElute column. Let column stand for 1-2 minutes.
8. Close MinElute column, centrifuge for 1 minute at 10,000 X g.

9. Store DNA at -20°C.
Protocol 5.6 Gel Extraction of Electrophoretically Separated DNA

1. Cut DNA band of interest from gel, minimizing gel excised.
2. Weigh gel in zeroed tube.
3. Add 3X volume of Buffer QG to gel (1µg of buffer to 1mg of gel) for gels under 2%. For gels above 2% add 6X volume of Buffer QG to gel. If gel mass is above 400 mg, split sample into tubes of no more than 400 mg.
4. Place tube with buffer and gel in 50°C H₂O bath to dissolve gel. Vortex tube every 2 minutes, total incubation time should be about 10 minutes. Extend incubation if necessary until gel is fully dissolved.
5. After gel is fully dissolved check the color of the buffer. If buffer is the original yellow color (color prior to incubation) proceed to step six. If the color of the buffer has changed from orange or violet add 10µL of 3M sodium acetate, pH 5.0 and mix. The color of the solution should change back to yellow.
6. Add one gel volume of isopropanol to sample (gel weight 400mg add 400µL) and mix by inverting tube a few times.

7. Add sample to MinElute column (make sure column is in a collection tube). Maximum volume of MinElute column is 800µL, if samples exceed 800µL repeat step 7 and 8 until all sample has been applied to column.

8. Close MinElute column, centrifuge for 1 minute at 10,000 X g, and discard flow through. Reuse collection tube for step 9.

9. Add 500µL of Buffer QG to the MinElute column.

10. Close MinElute column, centrifuge for 1 minute at 10,000 X g, and discard flow through. Reuse collection tube for step 11.

11. Add 750µL of Buffer PE to the MinElute column, let stand 2-5 minutes.

12. Close MinElute column, centrifuge for 1 minute at 10,000 X g, and discard flow through. Reuse collection tube for step 13.

13. Centrifuge for 1 minute at 10,000 X g, and discard flow through. Discard collection tube and place MinElute column in a new collection tube.
14. Add 10μL of Buffer EB (10mM Tris·Cl, pH 8.5) or RNase Free H₂O to the membrane of the MinElute column. Let column stand for 1-2 minutes.

15. Close MinElute column, centrifuge for 1 minute at 10,000 X g.

16. Store DNA at -20°C.
Protocol 5.7 DNA Concentration

1. Utilize 10mM Tris·Cl pH 7.5 to zero spectrophotometer.
2. Dilute DNA 1:50 to 1:1000 in 10mM Tris·Cl pH 7.5, for absorbance values to be accurate the absorbance should be between 1.0- 0.15.
3. Measure $A_{260}$.
4. $[\text{DNA}]\mu g/mL = 50 \times A_{260} \times \text{dilution factor (1:100 = 100)} \times \text{Light path (cm)}$.
5. DNA yield $\mu g = [\text{DNA}]\mu g/mL \times \text{volume sample mL}$. 
Protocol 5.8 DNA Purity ($A_{260}/A_{280}$)

1. Utilize 10mM Tris·Cl pH 7.5 to zero spectrophotometer.
2. Dilute DNA 1:100 to 1:1000 in 10mM Tris·Cl pH 7.5, for absorbance values to be accurate the absorbance should be between 1.0-0.15.
3. Measure $A_{260}$ and $A_{280}$.
4. Pure DNA has a $A_{260}/A_{280}$ ratio ranging from 1.9-2.3.
Protocol 5.9 DNA Sequencing Reactions of PCR Products

1. Purify PCR reactions by electrophoresis.
2. Isolate DNA from gel.
3. Calculate DNA concentrations and purity.
4. Calculate minimum amount of DNA needed in ng (100 ng/μL maximum concentration) = length of PCR product (bp)/5.0.
5. Add the calculated amount of DNA to sequencing tubes.
6. Add 0.2μL of 20p mole/μL forward and reverse primers to sequencing tubes.
7. Bring volume of reaction to 18μL with 10mM Tris, pH 9.0 or DNase free H2O.
Protocol 5.10 TOPO Subcloning of Blunt end PCR products

into ZeroBlunt4PCR-TOPO Vector

1. Prepare fresh PFX PCR reaction.
2. Analyze products by gel electrophoresis ensure only one band.
   If more than one band purify band of interest.
3. Add the following to a PCR tube:
   - Fresh PCR reaction 0.5 to 4μl
   - Topo salt solution 1μl
   - Sterile water add to final volume of 5μl
   - TOPO vector 1μl
   - Final Volume 6μl
4. Mix reaction gently and incubate at room temperature for 5-30 minutes (22-23°C).
5. Place the reaction on ice.
6. Proceed to transformation.
Protocol 5.11 Transformation of TOPO Vector into Chemically Competent Cell

1. Set up 42°C water bath.
2. Warm S.O.C medium to room temperature.
3. Warm two selective plates (LB 50µg/ml kanamycin or ampicillin) to 37°C for 30 minutes.
4. Thaw one vile of One Shot TOP10 chemical competent cells on ice per transformation.
5. Add 2µl of TOPO reaction (from step 5) to one vial of cells and mix gently (no pipetteing).
6. Incubate on ice for 5-30 minutes (incubation times seem not relevant to transformation optimization).
7. Incubate cells for 30 seconds at 42°C without shaking.
8. Place tubes on ice.
10. Incubate tubes horizontally at 37°C for 1 hour with rotation (200rpm).
11. Spread 10-50µl of transformation on prewarmed plates. For small volumes 20µl of S.O.C medium can be added prior to spreading to ensure uniform distribution. Two different concentrations should be plated to ensure well separated colonies.
12. Incubate plates at 37°C. Ampicillin plates take 8 hours and kanamycin plates incubate overnight.

13. Good transformations should yield several hundred colonies.

14. Pick ten well separated colonies and individual culture overnight at 37°C with rotation (200rpm) in 15ml of LB with 50-100μg/ml of ampicillin or 50μg/ml kanamycin.

15. Inoculate a slant of LB with 50-100μg/ml of ampicillin or 50μg/ml kanamycin and incubate overnight before storage at 4°C or glycerate and freeze cells at -80°C.

16. Proceed to plasmid isolation.

**Transformation Control**

2. Set up 42°C water bath.

3. Warm S.O.C medium to room temperature.

4. Warm two selective plates (LB 100μg/ml ampicillin) to 37°C for 30 minutes.

5. Thaw one vile of One Shot TOP10 chemical competent cells on ice per transformation.
6. Add 1µl of pUC19 10pg/µl to thawed cells.

7. Incubate on ice for 5-30 minutes (incubation times seem not relevant to transformation optimization).

8. Incubate cells for 30 seconds at 42°C without shaking.

9. Place tubes on ice.

10. Transfer 250µl of S.O.C. medium to tube.

11. Incubate tubes horizontally at 37°C for 1 hour with rotation (200rpm).

12. Add 10µl of transformation to 20µl of S.O.C. medium, spread on prewarmed plates.

13. Incubate plates at 37°C. Ampicillin plates take 8 hours or overnight.

14. Good transformations should yield several hundred colonies.
Protocol 5.12 Plasmid Isolation from *E. coli*

1. Make sure RNase A is added to Resuspension Buffer (R3) and was stored properly at 4°C. Confirm 100% ethanol was added to Wash Buffer (W9) and Wash Buffer (W10). Check Lysis Buffer (L7) for precipitate, if precipitate is present heat at 60°C until dissolved.

2. Pellet 1-5μl of overnight culture of transformants.

3. Decant media from pellet.

4. Resuspend pellet in 250μl of Resuspension Buffer (R3), making sure no clumps remain (pipette up and down).

5. Add 250μl of Lysis Buffer (L7) and mix gently by inverting tube 5 times (no vortexing), if necessary warm buffer to 37°C until salt dissolves (do not shake).

6. Incubate for 5 minutes at room temperature (Do not exceed 5 minutes).

7. Add 350μl of Precipitation Buffer (N4) and mix immediately by inverting tube until the mixture is homogeneous (no vortexing).

8. Centrifuge at 12000 X g for 10 minutes at room temperature.

9. Transfer supernate into spin column with wash tube, centrifuge at 12000 X g for 1 minutes at room temperature, and discard flow through.
10. Add 500μl of Wash Buffer (W10) to column, incubate for 1 minute at room temperature, centrifuge at 12000 X g for 1 minutes at room temperature, and discard flow through.

11. Add 700μl of Wash Buffer (W9) to column, centrifuge at 12000 X g for 1 minutes at room temperature, and discard flow through.

12. Centrifuge at 12000 X g for 1 minutes at room temperature and discard flow through.

13. Place spin column in recovery tube and add 75μl of TE Buffer to the center of column.

14. Incubate for 1 minute at room temperature and centrifuge at 12000 X g for 2 minutes at room temperature.

15. Analyze DNA for concentration by taking A260, purity by assessing the A260/A280 ratio (under 1.8 is good), and by restriction analysis and/or PCR to confirm integration of product of interest into TOPO vector.
Protocol 5.13 PCR Verification of TOPO Subcloning

1. Mix the following in a PCR tube:

   23.00μL Supermix

   0.50μL isolated plasmid

   0.75μL 20pmole/μL M13 forward (-20) primer

   0.75μL 20pmole/μL M13 reverse primer

**TOTAL VOLUME** 25μL

2. Use the following PCR program:

   Initial denaturing 2 minutes at 94°C

   35 cycles of the following:

   Denaturing 20 seconds at 94°C

   Annealing 30 seconds at 55°C

   Extension 40 seconds at 72°C

   Final extension 5 minutes at 72°C
3. Analyze product via agarose gel electrophoresis. Expected band size is 162bp + length of predicted insert.

4. Store reactions at -20°C until use.
1. Prior to sequencing plasmid DNA must be isolated from overnight bacterial culture and then subjected to verification of subcloning.

2. Add 1μg of plasmid DNA to a 500μl screw cap vial.

3. Add 8pmole of M13 forward or reverse primer (not both).
   Primers are also available from sequencing facilities.

4. If utilizing in house M13 primers bring reaction volume to 18μl with molecular biology grade water. If utilizing sequence facility supplied primers bring the reaction volume to 10μl with molecular biology grade water. If reaction exceeds final volume before the addition of water then lyophilize sample and reconstitute with molecular biology grade water to indicated final reaction volume.

5. Overnight samples to DNA sequencing facilities, no refrigeration is necessary for DNA samples.

6. Blastn results to confirm subcloning.
Appendix 2: Immunohistochemistry

Scott Leddon

Department of Biological Sciences

State University of New York College at Brockport

Brockport, New York

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### Appendix 2: Immunohistochemistry

#### Appendix 2.1: ICC Staining of the Zebrafish Gastrointestinal Tract

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<td>206-209</td>
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<td>Middle</td>
<td>100X</td>
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<tr>
<td>Posterior</td>
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<td>214-216</td>
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<td>Posterior</td>
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#### Appendix 2.2: ICC and Neuronal Cell Body Dual Staining of the Zebrafish Gastrointestinal Tract

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<thead>
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<th>Kit and HU C/D Dual Immunohistochemistry</th>
<th>Longitudinal Section of Gastrointestinal Tract</th>
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<td>40X</td>
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<td>Posterior</td>
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<td></td>
<td>222</td>
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<tr>
<td>Posterior</td>
<td>40X</td>
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Appendix 2.3: ICC and Neuronal Process Dual Staining of the Zebrafish Gastrointestinal Tract

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<th>Kit and α-Tubulin Dual Immunohistochemistry</th>
<th>Mag.</th>
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<tr>
<td>Longitudinal Section of Gastrointestinal Tract</td>
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<tr>
<td>Anterior</td>
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Appendix 2.4: ICC and Smooth Muscle Dual Staining of the Zebrafish Gastrointestinal Tract

<table>
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<th>Kit and Desmin Dual Immunohistochemistry</th>
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<tbody>
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<td>Longitudinal Section of Gastrointestinal Tract</td>
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<td>Middle</td>
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<td>Posterior</td>
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Appendix 2.5: Immunohistochemistry Controls of the Zebrafish Gastrointestinal Tract

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<td>CY5 2° Ab Bleed Through into CY3 Channel</td>
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Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
20X Anterior, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
20X Anterior, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
40X Anterior, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
40X Anterior, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
20X Middle, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
20X Middle, CY3 Filter
Anti-kit Immunohistochemistry
Zebrfish Gastrointestinal Tract
20X Middle, CY3 Filter
Anti-kit Immunohistochemistry
Zebrfish Gastrointestinal Tract
20X Middle, CY3 Filter
Anti-kit Immunohistochemistry
Zebrfish Gastrointestinal Tract
20X Middle, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
20X Middle, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
40X Middle, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
40X Middle, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
40X Middle, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
40X Middle, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
100X Middle, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
20X Posterior, CY3 Filter
Anti-kit Immunohistochemistry
Zebrasfish Gastrointestinal Tract
20X Posterior, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
20X Posterior, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
40X Posterior, CY3 Filter
Anti-kit Immunohistochemistry

Zebrafish Gastrointestinal Tract

40X Posterior, CY3 Filter
Anti-kit Immunohistochemistry

Zebrafish Gastrointestinal Tract

40X Posterior, CY3 Filter
Anti-kit Immunohistochemistry
Zebrafish Gastrointestinal Tract
80X Posterior, CY3 Filter
Anti-kit and Anti-HU C/D Immunohistochemistry

Zebrafish Gastrointestinal Tract

20X Anterior, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-HU C/D, bottom)
Anti-kit and Anti-HU C/D Immunohistochemistry

Zebrafish Gastrointestinal Tract

40X Anterior, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-HU C/D, bottom)
Anti-kit and Anti-HU C/D Immunohistochemistry

Zebrafish Gastrointestinal Tract

20X Middle, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-HU C/D, bottom)
Anti-kit and Anti-HU C/D
Immunohistochemistry

Zebrafish Gastrointestinal Tract

40X Middle, Cy3 Filter (Anti-kit, top),
Cy5 Filter (Anti-HU C/D, bottom)
Anti-kit and Anti-HU C/D
Immunohistochemistry

Zebrafish Gastrointestinal Tract

20X Posterior, CY3 Filter (Anti-kit, top),
CY5 Filter (Anti-HU C/D, bottom)
Anti-kit and Anti-HU C/D Immunohistochemistry

Zebrafish Gastrointestinal Tract

40X Posterior, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-HU C/D, bottom)
Anti-kit and Anti-α-tubulin Immunohistochemistry

Zebrafish Gastrointestinal Tract

20X Anterior, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-α-tubulin, bottom)
Anti-kit and Anti-α-tubulin Immunohistochemistry

Zebrafish Gastrointestinal Tract

40X Anterior, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-α-tubulin, bottom)
Anti-kit and Anti-\(\alpha\)-tubulin Immunohistochemistry

Zebrafish Gastrointestinal Tract

20X Middle, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-\(\alpha\)-tubulin, bottom)
Anti-kit and Anti-α-tubulin Immunohistochemistry

Zebrasfish Gastrointestinal Tract

40X Middle, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-α-tubulin, bottom)
Anti-kit and Anti-α-tubulin Immunohistochemistry

Zebrafish Gastrointestinal Tract

20X Posterior, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-α-tubulin, bottom)
Anti-kit and Anti-α-tubulin Immunohistochemistry

Zebrafish Gastrointestinal Tract

40X Posterior, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-α-tubulin, bottom)
Anti-kit and Anti-desmin Immunohistochemistry

Zebrafish Gastrointestinal Tract

20X Anterior, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-desmin, bottom)
Anti-kit and Anti-desmin Immunohistochemistry

Zebrafish Gastrointestinal Tract

40X Anterior, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-desmin, bottom)
Anti-kit and Anti-desmin Immunohistochemistry

Zebrafish Gastrointestinal Tract

20X Middle, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-desmin, bottom)
Anti-kit and Anti-HU C/D Immunohistochemistry

Zebrafish Gastrointestinal Tract

40X Middle, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-desmin, bottom)
Anti-kit and Anti-desmin Immunohistochemistry

Zebrafish Gastrointestinal Tract

20X Posterior, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-desmin, bottom)
Anti-kit and Anti-desmin Immunohistochemistry

Zebrafish Gastrointestinal Tract

20X Posterior, CY3 Filter (Anti-kit, top), CY5 Filter (Anti-desmin, bottom)
CY3 2° Bleed Through Control
Immunohistochemistry

Zebrafish Gastrointestinal Tract

40X Distal, CY3 Filter (Anti-kit, top), CY5 Filter (bottom)
Anti-kit Cross Reactivity with Anti-desmin, HU-C/D, α-tubulin 2° Antibody Control
Immunohistochemistry

Zebrafish Gastrointestinal Tract

40X Distal, CY3 Filter (Anti-kit, top), CY5 Filter (bottom)
CY5 2° Bleed Through Control
Immunohistochemistry

Zebrafish Gastrointestinal Tract

40X Distal, CY3 Filter (top), CY5 Filter (Anti-desmin bottom)
Appendix 3: Molecular Biology

Scott Leddon

Department of Biological Sciences

State University of New York College at Brockport

Brockport, New York
Appendix 3: Molecular Biology

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<td>KitaR475</td>
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<td>Kitb Receptor (kitb) Primer Optimization</td>
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<td>Kitb Ligand (kitbl) Primer Optimization</td>
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<tr>
<td>Conformation of cDNA Insertion into Sequencing Vector</td>
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RNA Integrity and Uniform cDNA Synthesis

03/02/2006
Scott Leddon

Gel 1
1.2% FA 1X Agarose Gel
Goal: Determine if the integrity of total GI RNA is suitable for downstream applications. Degradation/lack of rRNA indicates compromised RNA integrity.

Results

<table>
<thead>
<tr>
<th>Sample Set</th>
<th>Total GI RNA</th>
<th>Expected banding patterns for 28S, 18S, and 18S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total GI RNA</td>
<td>No rRNA bands, indicates lack RNA contamination</td>
<td></td>
</tr>
</tbody>
</table>

Diagram: Gel electrophoresis showing bands at 28S rRNA, 18S rRNA, and other DNA markers.
RT-PCR

2/14/2006
Scott Leddon

Gel 1
1.0 mM MgSO₄
2.0% TAE 1X Agarose Gel

Goal: Temperature optimization of 18sRNA primer set

Results

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Optimal Annealing Temperature</th>
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</thead>
<tbody>
<tr>
<td>18sRNA02</td>
<td>Expected band 54.4°C - 48.1°C</td>
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</tbody>
</table>

Bioline Hyperladder IV
RT-PCR

03/08/2006
Scott Leddon
Gel 2
0.5 mM MgSO₄
2.5% TAE 1X Agarose Gel
Goal-Primer Temperature Optimization

Results

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Optimal Annealing Temperature</th>
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<tbody>
<tr>
<td>KilaR304</td>
<td>63.2°C – 54.4°C, 2 products – higher annealing temperature may be needed</td>
</tr>
<tr>
<td>KilaR157R1</td>
<td>62.7°C – 56.3°C</td>
</tr>
</tbody>
</table>

KilaR304
KilaR304
KilaR304
KilaR304
KilaR304
KilaR157R1
KilaR157R1
KilaR157R1
KilaR157R1
RT-PCR

03/08/2006
Scott Liddon

Gel 4
0.5 mM MgSO₄

2.5% TAE 1X Agarose Gel

Goal: Primer Temperature Optimization

Results

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<thead>
<tr>
<th>Primer Set</th>
<th>Optimal Annealing Temperature</th>
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<tr>
<td>F2_R17</td>
<td>62.7°C - 56.3°C</td>
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<tr>
<td>KtsR4A0</td>
<td>60.2°C</td>
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</table>

18sRNA62: Prior studies optimized annealing temperatures, but this study shows 18sRNA62 requires [MgSO₄] = 1mM

3.2°C 62.7°C 48.5°C 59.7°C 63.2°C

F2_R17 54bp

KtsR4A0 49.3°C

18sRNA62

exACTGene 100bp PCR DNA Ladder

18sRNA62 50.7°C

5.2°C 49.3°C 48.1°C 52.4°C 56.3°C 60.6°C 62.7°C 59.3°C 50.7°C

exACTGene 50bp Mini DNA Ladder
RT-PCR

03/08/2006
Scott Leddon
Gel 3
0.5 mM MgSO4
2.5% TAE 1X Agarose Gel
Gent-Primer Temperature Optimization

![RT-PCR Image]

**Results**

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Optimal Annealing Temperature</th>
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<tbody>
<tr>
<td>KisaR222</td>
<td>65.7°C - 66.8°C</td>
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<td>KisaR475</td>
<td>65.2°C - 66.3°C</td>
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<td>62.7°C - 63.1°C</td>
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<td>Zlsh_b1.2</td>
<td>63.2°C - 64.9°C</td>
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<tr>
<td>KisaR584</td>
<td>62.7°C - 63.1°C</td>
</tr>
<tr>
<td>F2_R13</td>
<td>48.5°C - 49.1°C</td>
</tr>
</tbody>
</table>
RT-PCR

05/02/2006
Scott Leddon

Gel 1

0.5 mM MgSO4
PCRSUPERMIX
2.5% TAE 1X Agarose Gel

Goal: Primer Temperature Optimization

Results

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Optimal Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>KiloR594</td>
<td>69.2°C - 60.5°C</td>
</tr>
<tr>
<td>KiloR475</td>
<td>55.4°C</td>
</tr>
<tr>
<td>ZF Biol 2</td>
<td>56.5°C - 55.1°C</td>
</tr>
</tbody>
</table>
RT-PCR

04/26/2006
Scott Leddon

Gel 1
0.5 mM MgSO₄
PFX DNA Polymerase
2.5% TAE 1X Agarose Gel
Goal: Primer Temperature Optimization

Results

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Optimal Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kitbl.458</td>
<td>62.3°C - 64.3°C</td>
</tr>
<tr>
<td>Kitbl.470</td>
<td>62.3°C - 64.3°C</td>
</tr>
<tr>
<td>Kitbl.472</td>
<td>62.3°C - 64.3°C</td>
</tr>
<tr>
<td>Kitbl.475</td>
<td>62.3°C - 64.3°C</td>
</tr>
<tr>
<td>Kitbl.564</td>
<td>62.3°C - 64.3°C</td>
</tr>
<tr>
<td>Kitbl.450</td>
<td>62.3°C - 64.3°C</td>
</tr>
</tbody>
</table>

Notes:
- Primer set should be avoided - many nonspecific bands
RT-PCR

02/20/2006
Scott Leddon

Gel 1
1.0 mM MgSO₄
2% TAX 1x Agarose Gel

Goal:Temperature optimization of kith234 primer set.

Results

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Optimal Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>kith234</td>
<td>Expected band 63.2°C – 48.1°C</td>
</tr>
</tbody>
</table>
RT-PCR

02/24/2006
Scott Leddon

Gel 2
1.0 mM MgSO4
2.5% TAE 1X Agarose Gel

Goal: Temperature optimization of kitbr118 and kitbr50 primer set.

Results

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Optimal Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kitbr50</td>
<td>Failed at all temperatures, bands not appropriate sizes</td>
</tr>
<tr>
<td>Kitbr118</td>
<td>Expected bands 52.2°C - 62.3°C</td>
</tr>
</tbody>
</table>
RT-PCR
03/08/2006
Scott Leddon
Gel 1
0.5 mM MgSO₄
2.5% TAE 1X Agarose Gel
Goal: Primer Temperature Optimization

Results

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Optimal Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>KitbR149r1</td>
<td>62.2°C - 58.3°C</td>
</tr>
<tr>
<td>KitbR280</td>
<td>62.7°C - 55.3°C</td>
</tr>
</tbody>
</table>
RT-PCR

02/24/2006
Scott Leddon

Gel 1
1.0 mM MgSO₄

2.5% TAE 1X Agarose Gel

Goal - Temperature optimization of kita650 and kita470 primer set.

Results

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Optimal Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>kita650</td>
<td>Expected band 60.4°C - 48.1°C</td>
</tr>
<tr>
<td>kita470</td>
<td>Expected band 51.4°C - 49.5°C</td>
</tr>
</tbody>
</table>
RT-PCR

03/08/2006
Scott Leddon
Gel 4
0.5 mM MgSO₄
2.5% TAE 1X Agarose Gel
Goal: Primer Temperature Optimization

Results

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Optimal Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>F2_R17</td>
<td>62.7°C - 56.3°C</td>
</tr>
<tr>
<td>Kitah440</td>
<td>63.2°C</td>
</tr>
<tr>
<td>18srRNA62</td>
<td></td>
</tr>
</tbody>
</table>

18srRNA62: Prior studies optimized annealing temperatures, but this study shows 18srRNA62 requires [MgSO₄] = 1 mM
RT-PCR

05/16/2006
Scott Leddon

Gel 1

0.5 mM MgSO₄

PFX DNA Polymerase

2.5% TAE 1X Agarose Gel

Goal - Rerun of cloning primers for poster and publication
RT-PCR

03/14/2006
Scott Leddon
Gel 1

0.5 mM MgSO₄
2.5% TAE 1X Agarose Gel

Goal: PCR for DNA sequencing

Results

All primer sets yielded expected sized products for each target. Bands cut from gel and eluted with qiaquick mini elute kit.
RT-PCR

05/03/2006
Scott Leddon

Gel 1

0.5 mM MgSO₄

PFX DNA Polymerase

2.5% TAE 1X Agarose Gel

Goal: Amplification of targets for insertion into TOP4PCRBlunt Vectors for sequencing

Results

<table>
<thead>
<tr>
<th>Sample Set</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kbiol 550</td>
<td>Proper size band</td>
</tr>
<tr>
<td>Kbiol 470</td>
<td>Proper size band</td>
</tr>
<tr>
<td>All Other</td>
<td>Need to be rerun with more DNA</td>
</tr>
</tbody>
</table>
RT-PCR

05/03/2006

Scott Leddon

Gel 2 (rerun gel 1)

0.5 mM MgSO₄

PFX DNA Polymerase

2.5% TAE 1X Agarose Gel

Goal: Amplification of targets for insertion into TOPO4PCRBlunt Vectors for sequencing

Results

Sample Set

ZF_btl_2
All Other

Comments

Proper size band
No bands
RT-PCR
05/08/2006
Scott Leddon

Gel 1
0.5 mM MgSO₄
PFX DNA Polymerase
2.5% TAE 1X Agarose Gel

Goal: Amplification of targets for insertion into TOPO4PCRBlunt Vectors for sequencing

Results

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kibh234</td>
<td>52°C amplified optimally and used for TOPO cloning</td>
</tr>
<tr>
<td>Kibh485</td>
<td>52°C amplified optimally and used for TOPO cloning</td>
</tr>
<tr>
<td>All Other</td>
<td>52°C amplified optimally and used for TOPO cloning</td>
</tr>
</tbody>
</table>
RT-PCR

05/10/2006
Scott Leddon

Gel 1
0.5 mM MgSO₄

PCR SUPERMIX

2.5% TAE 1X Agarose Gel
Goal: Gel was run on TOPO4PCR Blunt
Vectors with insert. Primers were M13

Results

<table>
<thead>
<tr>
<th>Sample Set</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Sample</td>
<td>Proper size band for 55°C</td>
</tr>
</tbody>
</table>

exACTGene
100bp PCR DNA Ladder

Clone 10: Clone 1: Clone 3: Clone 4: Clone 5: Clone 6: Clone 7: Clone 8: Clone 9: Clone 10: Clone 11:
Conformation of Insert for Sequencing

05/07/2006
Scott Ledden

Gel 1
0.5 mM MgSO₄
PCRSUPERMIX
2.5% TAE 1X Agarose Gel

Goal-Gel was run on TOPO4PCRBunt Vectors with insert. Primers were M13. To calculate expected PCR product size from M13 priming of TOPO4PCRBunt Vectors add 162bp to the size of the insert.

Results

<table>
<thead>
<tr>
<th>Sample Set</th>
<th>Optimal Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Sample</td>
<td>Proper size band for 59°C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>82.3°C – 49.9°C</th>
</tr>
</thead>
</table>