Winter 12-2017

Implantation of Nanocellulose in the Zebrafish Model

Hanna J. Anderson
University of Maine

IMPLANTATION OF NANOCELLULOSE IN THE ZEBRAFISH MODEL

by

Hanna J. Anderson

A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Bioengineering)

The Honors College

University of Maine

December 2017

Advisory Committee:
Dr. Paul J. Millard, Advisor, Associate Professor, Dept. of Biological Engineering
Dr. Con S. Sullivan, Assistant Professor, Dept. of Molecular and Biomedical Sciences
Dr. Michael Mason, Professor, Dept. of Biological Engineering
Dr. Caitlin Howell, Assistant Professor, Dept. of Biological Engineering
Dr. David S. Gross, Associate Professor, Honors College Preceptor

ABSTRACT

The number of novel materials for use in biomedical implantation is expanding rapidly, increasing the success rates of implant procedures. Nanocellulose is being assessed as a sustainable and biocompatible material, offering an alternative to conventional polymer or metal designs with the appropriate structure for potential tissue integration. In this research, the capacity of cellulose nanofibers to serve as biomedical implants is assessed through examination of immune responses of transgenic zebrafish, utilizing bright field and confocal fluorescence microscopy. Methods for creating microincisions for the implantation of dense cellulose nanofiber shards in the zebrafish model were explored, and a surgical protocol was developed, along with an apparatus to aid with the procedure. Experiments suggest that nanocellulose implants induce slightly more neutrophil migration to the wound site than the injury itself, although more data are required to prove statistical significance. Integration of the nanocellulose implants also appeared to occur, although low implant retention rate rendered these experiments inconclusive.

TABLE OF CONTENTS

I. INTRODUCTION

Fish Tagging Procedures

The application of physical fish tagging devices is crucial to the advancement of fisheries science and studies of fish behavior. These devices can be used for species identification and documentation of migratory patterns. Several attachment options are available, depending on the intent of study. These fall into two categories: External, which include plastic buttons and discs, metal straps, and plastic or metals rods with various anchor methods; and internal, which include microtags, coded wire, and several types of tags that utilize sonar and radio frequencies (1).

Tagging procedures range in difficulty depending on the type and location of attachment. External tags that are not self-piercing are usually attached with a punch tool, needle, or pliers. Internal tags are more invasive and can be applied through microincision, injection, or larger-scale surgical implantation for bigger species of fish. A widely implemented internal tag is the passive-integrated transponder (PIT), which is suitable for recapture scenarios to collect data of growth rate, activity patterns, and distance traveled by the fish from the point of release (2). A PIT consists of a microchip encased in glass that can be coded to provide identification of individuals that had been tagged and released (1,2). These tags are injected or inserted by incision into muscle or the body cavity, then activated by an electromagnetic field from a handheld reader (2). Several studies have been conducted on PIT tagging methods to optimize survival andretention, including one performed by Baras et. al where it was concluded that surgical implantation was more effective than injecting the PIT tags into juvenile tilapia

once they had reached the proper size (3). These techniques can be implemented to promote the success of other investigations of fish surgeries and tag implantations.

Medical Implants

The pool of materials utilized in implantation procedures is constantly expanding as new engineering methods evolve and novel substances are created. Myriad variations of polymers, alloys, and natural fibers constitute the scope of medical implants. Several considerations must be accounted for when choosing a biological implant material since the intended purpose of the implant dictates the material properties required for success. Biocompatibility, surface structure, mechanical strength, and biodegradability are some of the important factors that must be considered in the design of a medical implant.

As part of the design process, the inertness of a material should be considered, as unexpected reactions to an implant can lead to complications of ranging severity, and could even be fatal. Therefore, extensive testing is crucial before it can be concluded that introduction into the body will be safe. An inert material is not cytotoxic and can exist within normal physiology without inducing a negative chemical or biological reaction. Even though largely inert, an implant may be rejected as the body recognizes it as a foreign object and attempts to expel it. This can be mitigated by configuring the implant structure to promote integration into the area of interest. Each tissue type and material has a distinctive surface chemistry that contributes to rejection or assimilation when combined (4,5), so a marriage between the properties of both sides must be formed for an implant to stay in position.

If an implant is designed to remain intact for several years, the chosen material must withstand degradation caused by biological processes within the body. These include

erosion by fluid flow, environmental pH effects, and the level of stress applied to the implant during normal wear (4). Mechanical strength and elasticity of a material can supplement its durability, especially if the site of implantation is within a highly mobile region and is subject to increased force. Reinforcement can be provided through chemical and mechanical processes, such as heat-treatment and coating, of materials that prove to be biocompatible but are less stable and resilient than is required. This promotes customization of implant design for applications in which a stronger, less-biocompatible material can be coated with a more inert substance to gain the benefit of both.

Implants are also used to support tissue repair and can be designed to be bioresorbable, where the implant stays in place for a determined amount of time then is naturally degraded by the body. This eliminates the need for a separate removal surgery of the implant and reduces the risks associated with performing multiple operations on the same area (5,6). Implants of this nature aid the healing process by providing a platform that tissue cells can adhere to and integrate. As the implant gradually degrades, more cells infiltrate the affected area and replace it with native tissue. The shape and structure of an implant influence the degradation process: a denser material will take longer to break down, and other factors such as crystallinity, thermal history, and chemical composition also impact the rate of deterioration (5).

Nanocellulose

Technological and scientific advances have led to increased development of biomaterials suitable for implantation. The popularization of naturally-sourced materials has prompted researchers to explore alternative, more sustainable options. Nanocellulose is a readily-available material that has been found to be biologically inert, biodegradable,

and resilient. It is produced by applying chemical and mechanical processes to naturally abundant cellulose derived from various forms of life, including plants and algae (7,8). In these processes cellulose is extracted in its original form and its structure manipulated on the nanoscale, creating a product with versatile applications determined by its method of manufacture.

Nanocellulose is generally produced in three forms: cellulose nanocrystals (CNC), cellulose nanofibers (CNF), and bacterial nanocellulose (BNC). For CNC, chemical deconstruction is employed to isolate crystalline cellulose from amorphous regions of material, whereas CNF are created by finely grinding and enzymatically treating cellulose to create rod-like fibers with varying dimensions dependent on the technique of production (9). In contrast to both CNC and CNF, BNC is manufactured in bioreactors containing bacteria that synthesize and extrude the nanocellulose (9,10). The latter process is more expensive and time consuming, as the correct conditions must be met to ensure the success of the bacterial culture for large-scale production.

This study examines the potential of using dense CNF as a replacement for conventional polymer implant materials. Variations in structure including density, porosity, and specific shape of implant materials affect tissue integration, therefore optimization of these parameters is crucial for the improvement of existing implant formats. When suspended in an aqueous solution and then dehydrated, CNF can be fused into a solid form, creating a block of material. Containing numerous hydroxyl groups, CNF molecules create both intermolecular and intramolecular hydrogen bonds that promote cohesion and association (7,10). Due to heterogeneous fiber dimensions, CNF assemble into a semi-porous structure during the dehydration process (11), the extent of which is

dependent on the moisture content of the material. This porosity can increase cellular integration, permitting a more secure implant that remains inert when introduced into tissue.

Microincision Techniques

Surgeries on the microscale require precision and are difficult to complete successfully, even with the appropriate tools and techniques. Microincisions are performed in surgeries where deftness is crucial. Two examples include ocular surgery and neurosurgery, where manual skill and technology are merged to maximize precision. Common practice in corneal and cataract surgeries is phacoemulsification along with microincision, a process that includes an ultrasonic tip with aspiration and irrigation to maintain the interior fluids of the eye (12,13). Several techniques have been detailed during the quest for optimal accuracy with the fewest detrimental effects on the ocular tissue, and conventional instruments have been established despite some variation in gauge (12–15). Neurosurgery is similar in this regard and continued advances in research aim to improve the intricate operations.

Animal models may be subjected to surgeries in which microincisions are made, depending on the nature of the research; these may include such areas as dermatology, myology, immunology, microdissection, and others. The methods for creating a microincision vary with the field of study, but it is common to use a dermal laser or a microscalpel fashioned from stainless or carbon steel, titanium, or tungsten. In the work of Gerlach, Morales, and Wingert (16), tungsten needles were utilized to aid in the implantation of microbeads in embryonic zebrafish. The needles were used to create small incisions in which microbeads were inserted with the aid of a whisker/lash tool, resulting

in a minimally affected region of tissue. The current project examined the functionality and precision of tungsten microscalpels compared to carbon steel microscalpels when creating microincisions in the epidermis of the zebrafish.

The Zebrafish Model

During recent years, *Danio rerio* have become widely used in biomedical research due to their embryonic transparency and rapid breeding, producing up to 100-400 eggs per breeding pair in a week (17). Optical transparency persists until 5 days post-fertilization (dpf) in wild-type zebrafish, allowing straightforward examination of developmental processes and the intricate physiology of the fish. Zebrafish mirror more closely the biological processes of higher-level organisms than simple cultured cells and offer delayed nerve development in a more cost-effective animal model. Development of transgenic zebrafish lines has greatly improved certain experimentation techniques by permitting the evaluation of multiple biological parameters while producing large quantities of specimens.

The zebrafish model has been utilized in epidemiology studies due to distinct characteristics of its epidermis compared to that of mammals, most notably that it contains robust regenerative properties (17,18). Zebrafish epidermis consists of live cells throughout each layer, as opposed to mammalian epidermis, which is covered by a layer of dead cells (19). This contributes to rapid re-epithelialization, a process which has been shown to occur within 24 hours in the adult zebrafish (17,18). Richardson, et. al. conducted a study to assess the immune response of zebrafish when a tissue wound was inflicted (18). An injury was created for the observation of regeneration of epithelial cells and the inflammatory response to injury at the site of a large excision in the zebrafish tissue. The

resulting healing process was discovered to follow the same steps as adult mammalian wound repair, with the advantage of accelerated progress, an aspect that is beneficial for high-throughput studies of cutaneous injury repair.

Among myriad applications for investigative studies, the zebrafish is also an excellent model for the observation of immune responses. This is primarily due to the many advantages that zebrafish offer, including rapid development and the presence of both innate and adaptive immune responses, where activation of the innate response occurs within 30 hours post-fertilization (hpf) (17), and the adaptive response develops between 4-6 weeks post-fertilization (wpf) (20,21). This period between the two types of responses allows extended observation of the intricacies of the innate immune response of the model. In a study conducted by Mathias, et. al (22), MPO:GFP zebrafish were utilized to assess the *in vivo* inflammatory response after a wound was inflicted on the tail. High-resolution imaging during the immune response captured transgenic neutrophil migration to the site of injury starting at 6 hours post-injury (hpi), and evidence of neutrophil chemotaxis from the injury site toward the vasculature was shown during the resolution phase of inflammation (22).

During an immune response, neutrophils travel to areas of inflammation to phagocytose pathogens and other incidental microorganisms. Additional effects that this behavior may impart on the overall immune response are still being studied (23). In the case of this particular project, the migration of neutrophils toward the implanted nanocellulose was modeled to observe its biocompatibility. As the immune responses of zebrafish are in many ways comparable to those of mammals (17), observed cellular

behavior in this study is analogous to the potential immune response of other animals or humans toward nanocellulose in its solid form.

Objective

The main objective of this pilot research project was to utilize the zebrafish animal model system to assess the potential of dense CNF for use in medical implants. Previous work does not describe implantation studies of this exact nature, but techniques from several areas have been combined to inform this effort. One aspect of the project focuses on methods for implanting the novel nanocellulose material into zebrafish to evaluate the potential of this material for use in prosthetics in humans, as zebrafish generate similar immune responses that can be readily observed (17). This project holds considerable potential for the advancement of naturally-sourced implantable biomaterials due to the biocompatibility, durability, and availability of nanocellulose. Implantation in zebrafish required microincisions to be made and physical modification of the nanocellulose to permit insertion. A new approach for creating microincisions in juvenile zebrafish was explored. Since 96 hpf zebrafish are delicate and range only between 3.5-4.0mm in length (24), conventional techniques are rendered difficult to implement while maintaining the survival of the animal. After implantation, visualization and quantification of neutrophils migrating to the site of implantation and the observation of cellular integration of the CNF were conducted. Immune responses and other changes in the condition of surrounding tissue were monitored and recorded by bright-field and confocal fluorescence microscopy.

II. MATERIALS AND METHODS

CNF Manipulation and Shaping

Dense CNF material was graciously provided as a solid block by David Holomakoff, Dept. of Chemical and Biological Engineering at the University of Maine, To create thin wafers of CNF, two mechanical methods were attempted using the machinery in the Mechanical Engineering Technology laboratory at the University of Maine. After squaring the CNF block to ensure consistent measurements, the first method utilized a six-flute high helix carbide end mill with a 1.0in diameter set at 0.025-0.050mm thickness, which produced tightly coiled curls of CNF, resembling a spindle shape that ultimately proved too large to use as an implant in the zebrafish (*Figures 2.1-2.2*).

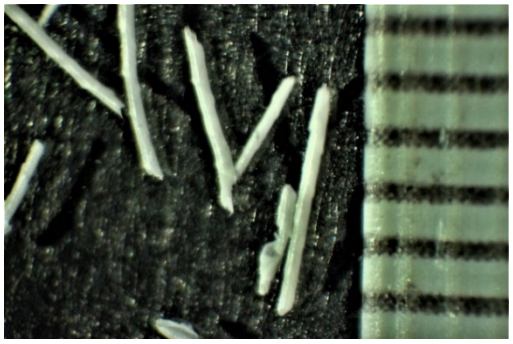


Figure 2.2. Spindles generated with end mill. (~0.05mm thickness)

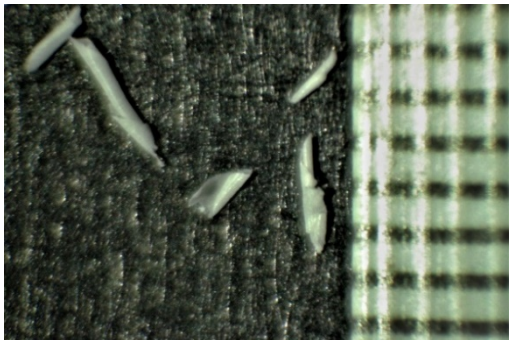


Figure 2.2. Spindles generated with end mill (~0.025mm thickness)

Since the intent was to create a 'flat' implant, the second method employed the use of a hand plane, which was used to successfully shave thin CNF strips from the original block (*Figures 2.3-2.4*). Digital calipers were used to identify strips of the appropriate thickness for further manipulation.

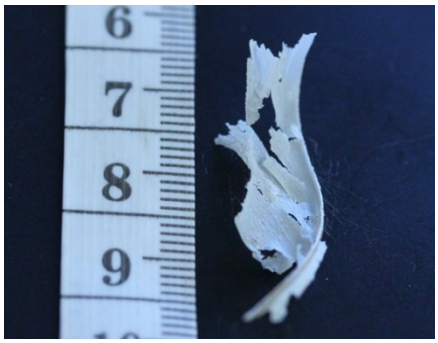


Figure 2.3. Shavings generated with hand plane (>0.05mm thickness)

Figure 2.4. Shavings generated with hand plane (<0.05mm thickness)

The original aim of the experiment was to compare the extent of inflammation and tissue integration exhibited by different shapes of CNF, therefore, the CNF strips were initially cut into three different shapes: squares, triangles, and rectangles (*Figures 3.1-3.3*).

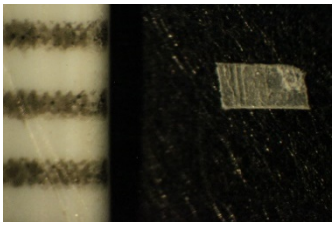


Figure 3.1. Rectangle CNF

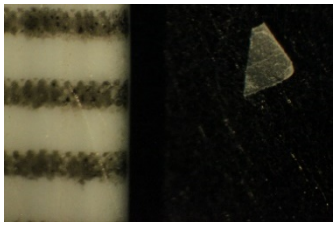


Figure 3.2. Triangle CNF

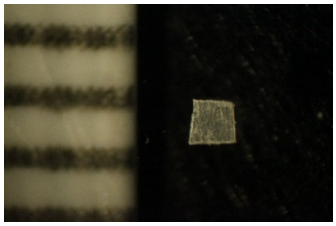


Figure 3.3. Square CNF

While the experiment was eventually reduced in scope to include only the square implants, the method of manufacture was the same among the three configurations. Using a stereomicroscope with a black stage as a backdrop (*Figure 3.4*), the CNF strips were held in place with forceps in the non-dominant hand and cut with an angled utility knife in the other. Shaping of the CNF was challenging, as the strips would split from the stress of the knife coupled with the thinness of the material. The shard would tear prematurely, creating inconsistent implant sizes that had to be discarded. It was found that focusing the pressure on the blade to create a clean chop produced the best results, rather than slicing the knife through the CNF.

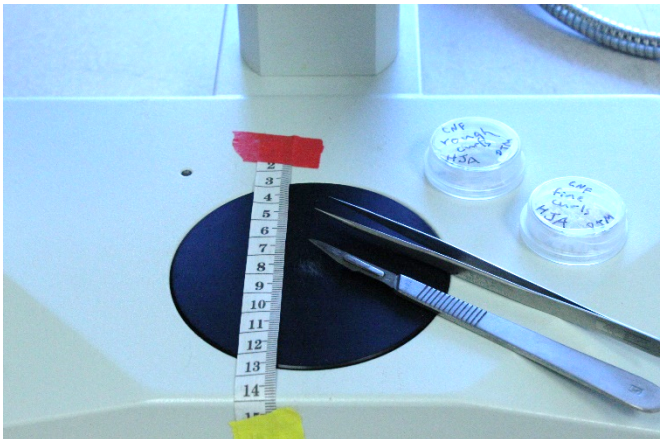


Figure 3.4. Complete shaping setup

Nanocellulose is prone to swelling in water due to the same reactive hydrogen bonds that dictate the self-associative properties of the material (10). For this reason, it was anticipated that the implants would swell *in vivo* amid the interstitial fluid of the zebrafish. To accommodate this, the implant size was reduced to approximately 0.50 x 0.50 x 0.025mm. An assessment of swelling was conducted by imaging a dry 1.0 x 1.0 x 0.025mm square of CNF, then leaving it in a petri dish of water overnight. The square was imaged again after removing excess water and the dry and wet dimensions were compared (*Figures 4.1-4.2*), showing a slight increase in size after soaking.

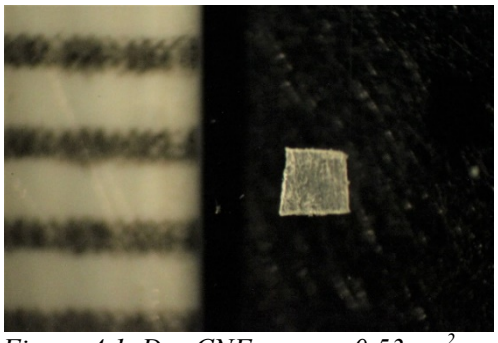


Figure 4.1. Dry CNF square, 0.53mm^2

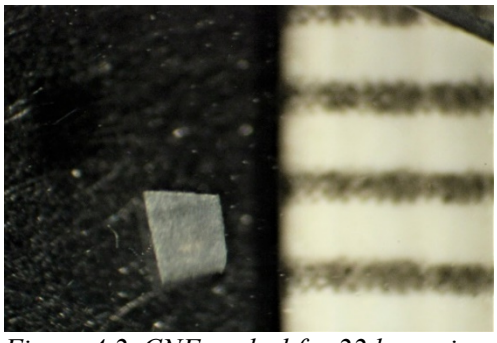


Figure 4.2. CNF soaked for 22 hours in deionized water, 0.68mm^2 (22% increase in area)

Further assessment could utilize a physiological buffer instead of water to represent swelling of the shard amidst the interstitial fluid of the fish. This experiment also indicated increased fragility in wet CNF, illustrating a potential complication that could arise following implantation in the zebrafish.

Zebrafish Transgenic Lines

This project utilized four lines of zebrafish: Tg(mpx:EGFP), Tg(fli1a:EGFP), Casper crossed with Tg(mpx:EGFP), and Casper crossed with Tg(fli1:EGFP). Tg(mpx:EGFP) *Danio rerio* is a transgenic line of zebrafish that expresses EGFP under control of a neutrophil-specific myeloperoxidase promoter, where EGFP with an SV40 polyadenylation site was inserted at the mpx ATG start site, also termed 'mpo' (25). The transgenic line, fli1 *Danio rerio,* expresses EGFP along its vasculature through the use of a promoter for *fli1* (*Figure 51*) (26).

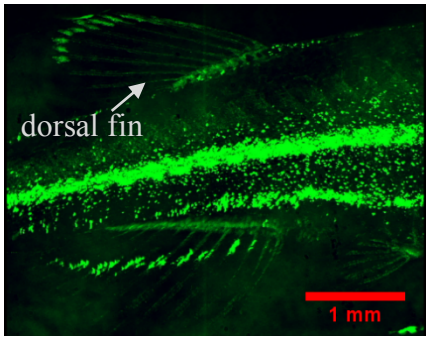


Figure 5.1. fli1 Danio rerio

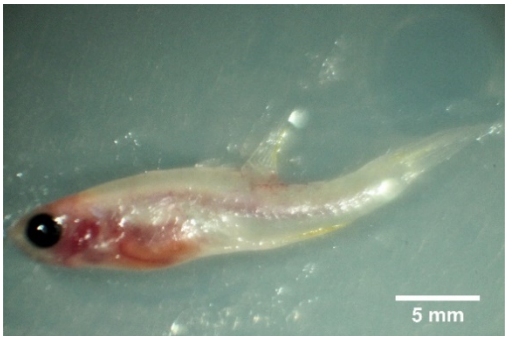


Figure 5.2. Euthanized Casper Danio rerio, 5 months old

These transgenic lines are similar to AB-wildtype zebrafish with the exception of the expression of fluorescent protein. The Tg(mpx:EGFP) line was developed specifically for use in immune response studies by Renshaw, et.al. at the MRC Centre for Developmental and Biomedical Genetics, University of Sheffield, Sheffield UK to provide a more efficient research method for imaging neutrophil migration (25). The Tg(fli1:EGFP) line was

developed for observation of blood vessel development in zebrafish by Lawson and Weinstein at the Laboratory of Molecular Genetics, NICHD, NIH in Bethesda, Maryland (26). Tg(mpx:EGFP) and Tg(fli1:EGFP) embryos were obtained from adult fish housed in the University of Maine zebrafish facility. For the purposes of this document, the Tg(mpx:EGFP) and Tg(fli1:EGFP) zebrafish lines are referred to as MPO and fli1, respectively.

Casper (*roy;nacre) Danio rerio* contain genetic mutations that prevent pigment formation, rendering the fish transparent into adulthood (*Figure 5.2*). This line of zebrafish was created through the combined effort of Richard Mark White and Anna Spessa et. al at the Dana Farber Cancer Institute, The Children's Hospital Boston, Massachusetts General Hospital and Harvard Medical School in Boston, Massachusetts to enhance the sensitivity of imaging of stem cells and transplanted tumor cells in adult fish (27). For the development of the Casper transgenic line, two previously discovered zebrafish mutations were combined: nacre (nac^{w2}), which are devoid of melanocytes that produce the striped appearance in wild-type fish; and roy orbison (roy^{a9}), which results in the deficiency of iridophores that provide reflective properties to the fish (27). The result of the cross is a line of zebrafish suitable for extensive organ observation in adult zebrafish that was formerly limited by pigmentation. The Casper adults utilized in breeding the MPO and fli1 crosses were also housed in the University of Maine zebrafish facility.

PTU Administration

To ensure tissue transparency throughout the maturation of the MPO and fli1 zebrafish groups, 1-phenyl 2-thiourea (PTU) was administered prior to development of pigmentation in the embryo and then as a continuous treatment thereafter to prevent

development of melanophores. Treatment during early embryogenesis is important because PTU inhibits the conversion of tyrosine to melanin, a mechanism which effectively blocks the formation of pigment but does not eliminate pigment that is already present (28). Following the protocol developed by Karlsson, von Hofsten, and Olsson (28), a 75μM PTU solution was prepared (Appendix B.1) and introduced to 24 hpf embryos (Appendix B.2), where about 200 fertilized eggs had been collected from each transgenic line the day before. This protocol was followed until the embryos reached 5 dpf, the age at which the yolk sac diminishes and the zebrafish have developed the necessary gut anatomy to eat. At this point, all larval fish were transferred to 2.75 liter tanks and daily feeding was initiated.

Due to the requirement of constant immersion in PTU, the fish could not be kept in the flow-through tank system that has been established in the secondary zebrafish facility to maintain a steady stream of water promoting oxygenation. To accommodate PTU treatment, the flow-through tanks were stoppered and mesh inserts, or 'baskets', were fabricated to allow the preservation of the fish and efficient daily changes of the solution (Appendix B.3). A previous experiment that required an immersion treatment used a series of plastic cups with mesh insets to contain the fish within the tanks for easy removal during solution changes. This new basket design was implemented instead to optimize the space that the zebrafish could traverse within the tank to promote optimum growth. Like many organisms, zebrafish growth is limited by the space in which they are enclosed, determined by both the tank volume and the population density of fish within the tank. To ensure the success of this experiment, the goal was to yield the largest zebrafish possible within a two-month period, which prompted the use of the basket inserts and feeding three-times daily.

It was crucial to change the PTU solution every day to maintain sufficient oxygen levels for the survival of the zebrafish, and a protocol was devised to reduce the risk of human exposure to the toxic chemical (Appendix B.4). The MPO and fli1 groups were maintained in this way until the zebrafish were 11 wpf, and it was concluded that populations were too small to continue the experiment using the PTU-treated fish. There are several factors that may have contributed to the high mortality during this treatment, including the stress on the fish from daily removal from water, or the enhanced growth of algae in the uncirculated water. Algal growth occurred very quickly from feed and fecal matter collecting in the fibers of the mesh baskets, which were difficult to clean while containing zebrafish. It was also challenging to avoid collecting fish when maneuvering the siphon or skimmer during tank cleanings because of the transparency of the zebrafish.

As Casper *Danio rerio* have become the preferred transparent zebrafish model, limited protocols exist describing the administration of PTU, particularly involving continuous treatment into adulthood. It is possible that the concentration of PTU used in this work, while suitable for short-term experiments, may have had deleterious effects with prolonged administration. More testing will be required to confirm this hypothesis. The zebrafish salvaged from the PTU-treated MPO and fli1 groups were completely transparent (*Figures 6.1.-6.2*), evidence that the regimen had been effective in its main purpose, however the remaining fish also appeared stunted in growth when compared to the Casper fish of the same age. This may have been caused by the extended PTU treatment, or possibly the reduced frequency of feeding in the fish fed twice daily instead of three times.

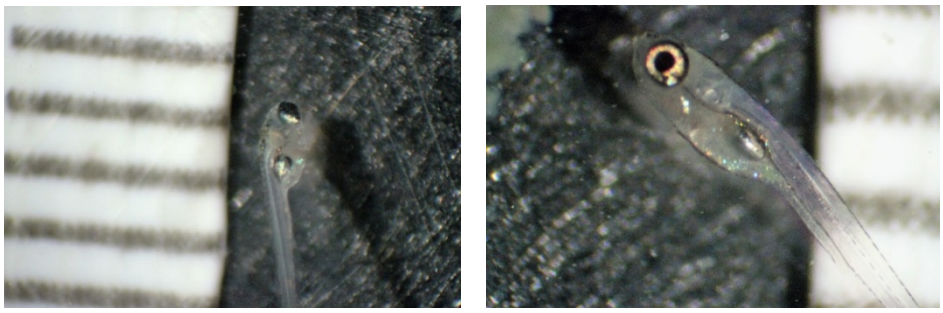


Figure 6.1. Fli1 Danio rerio *(mm)*

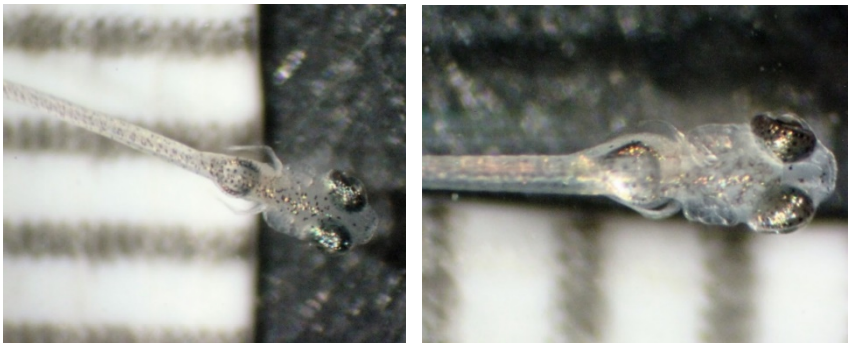


Figure 6.2. MPO Danio rerio *(mm)*

Tungsten Microscalpel Sharpening

Multiple options for a microincision instrument were considered, including commercial microscalpels, hypodermic needles, and sharpened tungsten wire. Tungsten is a durable transition metal that can be easily honed to a sharp point using electrolysis. Electrolysis is a process in which a current is passed through an electrolyte solution, mobilizing reacting materials between the electrode and the solution (29). If a conductive metal, such as a tungsten wire, is used as the anode, controlled decomposition can occur as electrons are withdrawn from appropriate metal atoms to create cations that dissolve into aqueous solutions. In this case, surface layers of the tungsten wire progressively decompose on the atomic level to create an acute point.

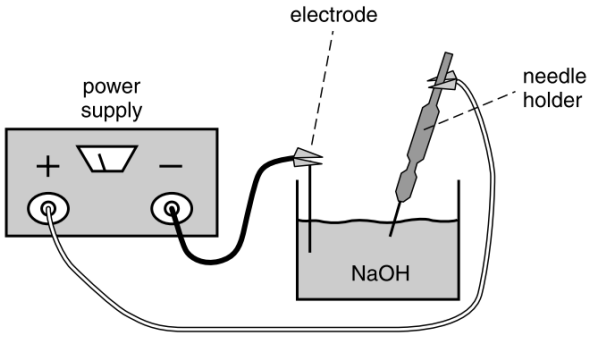


Figure 7.1. Electrolysis setup for tungsten decomposition. Reproduced from Ref [30].

Following the protocol described by Moore and Kennedy (30), coiled tungsten wire of 0.5mm diameter was cut into 10-15cm segments (Appendix B.5). The tip of the wire was placed in 1M NaOH and an electric current was applied to initiate electrolysis (*Figure 7.1*). The wire was positioned in the solution using several orientations to generate different tip shapes. For example, vertical submersion (perpendicular to the surface of the NaOH solution) yielded a straight, conical tip by raising the wire out of the solution in increments to create a gradual taper (*Figures 7.2-7.3*). The diameter of the tip could be adjusted by varying the length of time that the wire was submerged in the NaOH. A sharpened tip with a flat bottom edge was produced by creating a 90° bend in the wire approximately 1cm from the end and placing it on the meniscus of the NaOH solution (*Figure 7.4*). This procedure was inspired by the work of Conrad et. al where detailed studies were conducted on the fabrication of tungsten needles (31). When left at the surface of the solution, the wire is partially surrounded by the meniscus interface and degraded only where contact is established with the solution. To create a conical tip as well, the end of the wire was placed at a slight angle so that the furthermost point was submerged just under the surface of the meniscus, incorporating the previous effort for a gradual sharpened effect along with the flattened bottom edge.

Figure 7.2. Vertical submersion

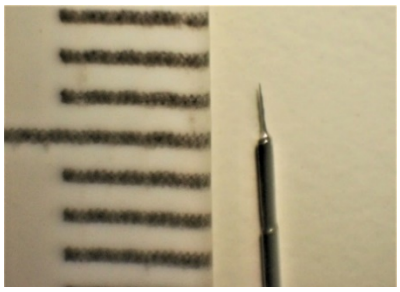


Figure 7.3. Vertical submersion, gradual withdrawal

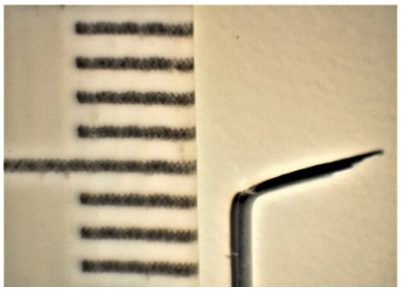


Figure 7.4. Meniscus, flat edge

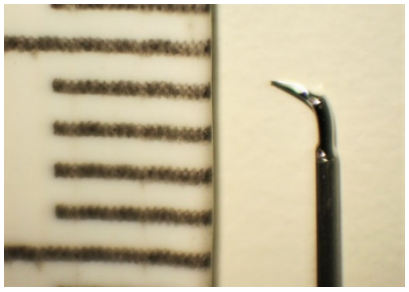


Figure 7.5. Meniscus/vertical submersion, hook

Several other variations were produced, including a hook-like version in which approximately 0.5cm of wire was bent at 90°, then the tip was positioned perpendicular to the surface of the NaOH solution and submerged for a greater length of time at a higher voltage than before. Near the bend, the angle between the solution interface and the tungsten wire was no longer perpendicular, resulting in a short, curved hook after most of the submerged wire had been dissolved (*Figure 7.5*). See Appendix A.7 for a catalog of the tungsten needles that were created.

Surgical Water Supply

To provide a constant water supply to the zebrafish and ensure proper oxygenation during the operation, an apparatus was constructed out of 80/20 T-slot aluminum, silicone tubing, and tube connectors. For a stable structure, the 80/20 aluminum was attached to a threaded stage with a 90° corner bracket and ¼ - 20 socket head cap screws (5/8” length).

Water was supplied by gravity and initiated by repeatedly adding system water to the reservoir of a 60mL syringe affixed to the top of the 80/20 aluminum. The flow of water was directed through a 10cm section of 3/32" ID, 5/32" OD silicone tubing, then split between two 4cm sections of the same silicone tubing using a plastic Y-connector (*Figure 8.3*). Customized plastic connectors were inserted into the outflow end of each 4cm section to direct the water flow into successive tubing segments. One of these connectors, labeled Connector 2, contained two identical 90° attachments arranged as in *Figure 8.4*. The other connector, Connector 3, contained two of the same 90° attachments as in Connector 2 as well as one straight attachment, arranged as in *Figure 8.5*. Each of these attachments on Connectors 2 and 3 was inserted into 35cm sections of 1/32" ID, 3/32" OD Tygon® tubing, totaling five separate tubes for the water to flow through.

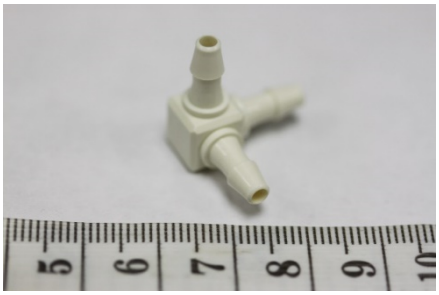


Figure 8.3. Connector 1

Figure 8.4. Connector 2

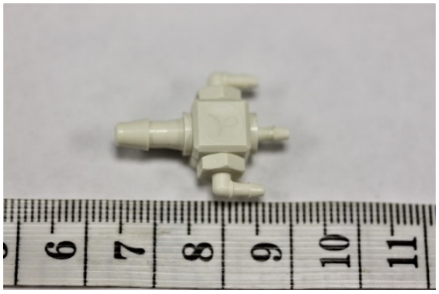


Figure 8.5. Connector 3

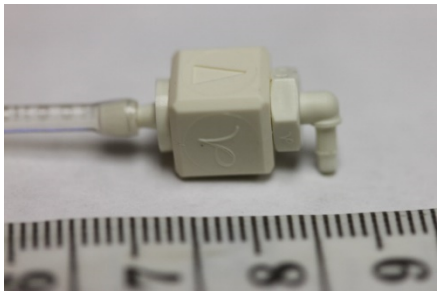


Figure 8.6. Connector 4

At the end of each 35cm section was another customized connector (*Figure 8.6*) that contained a single 90° attachment of a smaller diameter than in Connectors 2 and 3.

The system water exited the apparatus through this attachment to be directed onto the gills of the zebrafish at a steady rate. These five connectors were distributed evenly around the perimeter of an agar petri dish to coincide with the molded impressions (described below) and fixed in place with hot glue. The smaller diameter of Connector 4 reduced the volumetric flow rate of the water and allowed any overflow in the agar plate to be managed quickly with a vacuum aspirator and flask.

Reverse-Mold Agar Well

To establish a platform on which to perform the implantation, a reverse-mold well was designed using SolidWORKS, then 3D-printed with polylactic acid (PLA), a cheap thermoplastic commonly used for 3D extrusion. The main function of the reverse-mold well was to create a run-off system that would provide adequate drainage for the constant water supply running over the gills of the fish. As determined with the practice tests using 10.5 wpf zebrafish, it was extremely challenging to execute an incision because the thin layer of water on the epidermis deflected the blade and the fish would slide out of position. Therefore, the reverse-mold well was designed to also form a holding cell that would minimize displacement of the fish during surgery. The resulting molded piece consisted of a gradual protrusion that began at the tail position and increased in prominence as it approached the gill region (Fish Placement Zone, *Figure 9.4)*, eventually meeting a rectangular projection that jutted away from the main piece at the maximum point (Water Drainage Channel and Water Outlet Channel, *Figure 9.4*). When applied to a moldable material, prominence facing down, the result was a sloped impression to draw water away from the site of implantation in the tail area. This also prevented overflow by funneling incoming water into an exit channel.

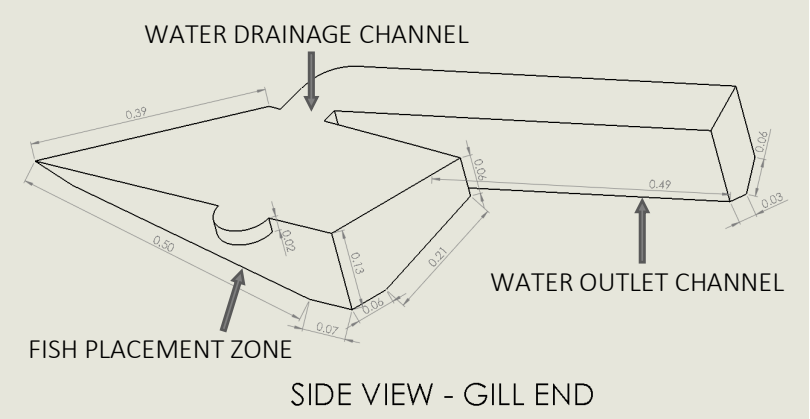


Figure 9.4. SolidWORKS rendering of reverse-mold agar well

Once the reverse mold had been fabricated and several had been printed (Appendix A.9), 2% bacteriological agar plates were poured and allowed to partially set. To increase the efficiency of the operation, five adult fish were intended to be situated on the agar at one time, so five reverse molds were placed in the gelatinous agar with the outflow channels facing the middle of the plate (*Figures 10.1-10.2*). After the agar had set completely, the reverse molds were carefully removed using forceps, and a hole was carved in the middle of the plate to connect the outflow channels. The pipette tip of a vacuum flask was applied by an assistant into the center of the carved portion to remove the water collected from all five impressions.

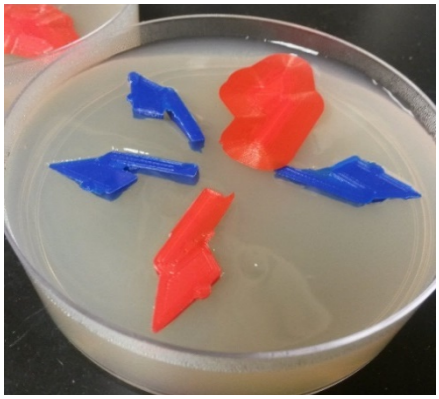


Figure 10.1. Reverse-mold applied to agar

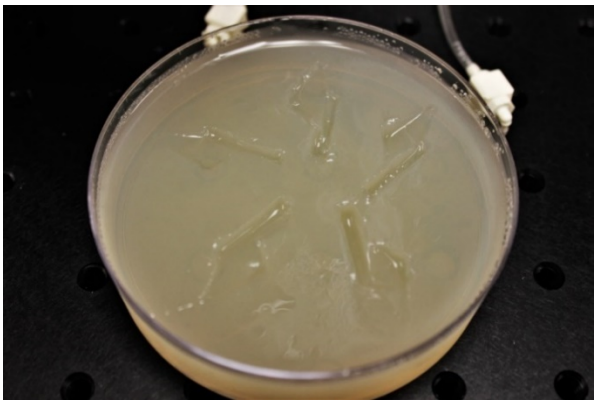


Figure 10.2. Reverse-mold removed

Microincision and Implantation

Before the operation took place, several practice rounds were conducted to determine the most efficient method for performing the procedure. The efficacy of the tungsten needles was compared to that of the microscalpels in different mounting configurations, using a micromanipulator to aid in the uniformity of the maneuver. Microincisions were first practiced on some of the 10.5 wpf Casper crosses with limited success. The ease of creating a clean cut was assessed, and it was found that the tungsten needles performed better when the procedure was done manually, but the microscalpels surpassed the tungsten when paired with the micromanipulator (*Figures 11.1-11.4*). Because uniformity of the incisions was the required to maintain the integrity of the experiment, it was decided that the latter setup would be utilized in the succeeding operations.

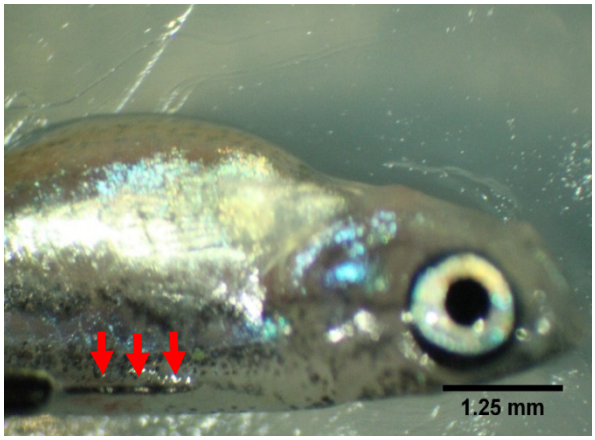


Figure 11.1. Red arrows indicate microincision performed with the vertical submersion, gradual-withdrawal tungsten needle (under epidermis)

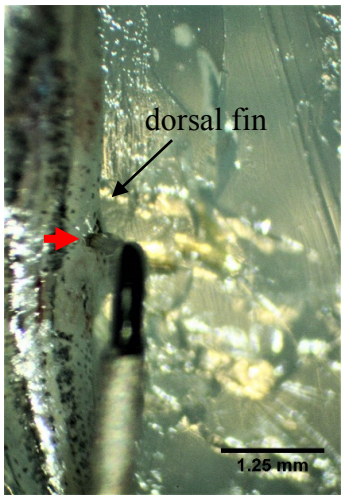


Figure 11.2. Red arrow: Tungsten hook inserted into dorsal line tissue

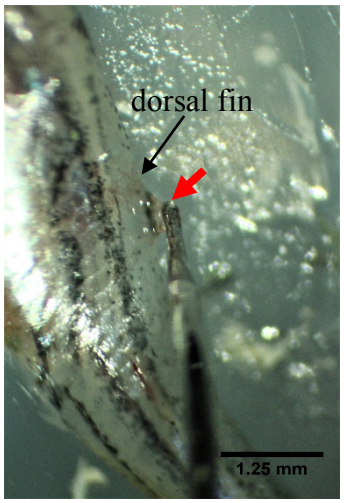


Figure 11.3. Red arrow: Angled tungsten needle lifting flap of epidermis

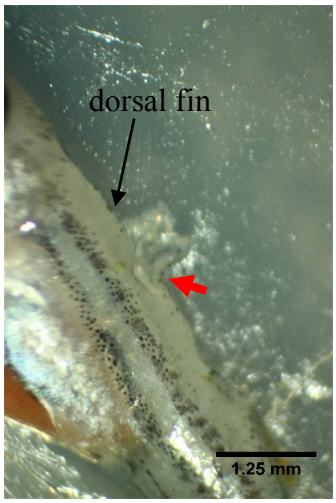


Figure 11.4. Red arrow: Pocket created by micromanipulator and 22.5° stab microscalpel

The second day of practice involved implantation and utilized 5 month-old Casper fish to provide a larger platform for determining the most efficient method. Using the previously established microscalpel/micromanipulator setup, several approaches were attempted to create an incision and then insert a square piece of CNF. It was discovered that the best technique was to use the vertical adjustment knob on the micromanipulator to move the 22.5° carbon steel microscalpel until it punctured the epidermis of the zebrafish about 0.5mm deep. Then, instead of producing a horizontal movement to create a large incision, the microscalpel was left in place. When the former method was attempted, the entire fish was dragged by the blade. By remaining stationary within the fish, the 22.5° microscalpel served as a guide to insert the CNF implant using the slightly bent tip of the 15° carbon steel microscalpel under the epidermis. This process worked well with the older Casper fish, and the implant was clearly visible (*Figures 11.5-11.6*).

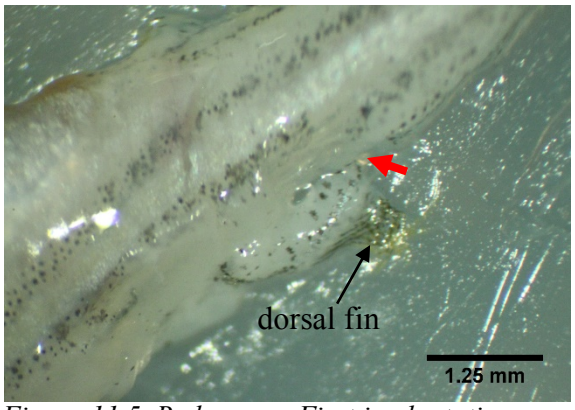


Figure 11.5. Red arrow: First implantation attempt using micromanipulator and manual insertion

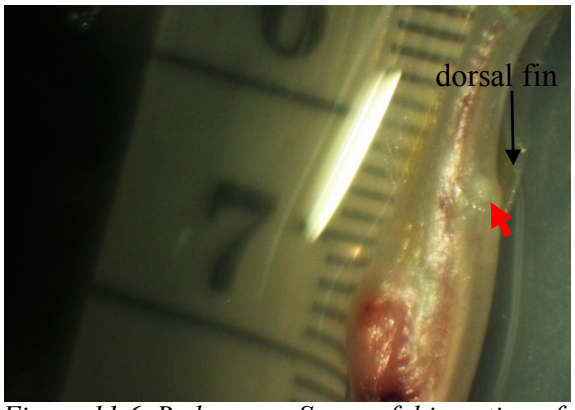


Figure 11.6. Red arrow: Successful insertion of square CNF implant ventral to the dorsal fin in 5 month- old Casper

The proposed experiment setup consisted of three trials: Incision-only, to serve as a negative control for observing natural response to an injury; Glass shard insertion, to serve as a positive control for observing the response to a known, inert material; and CNF shard insertion for the purpose of this experiment. During the operation, the Casper crosses were split evenly into the appropriate trials (*Figure 12.3*). There were enough fish of each line to fall within the 20-30 fish that were desired for each trial, 20 being the final count for the MPO crosses and 21 for the fli1 crosses, after the smallest fish were removed.

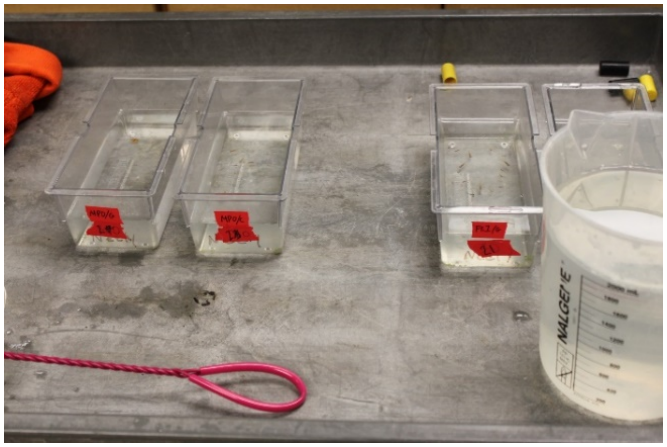


Figure 12.3. Zebrafish trial groups on standby for operation

The nanocellulose shards were placed in a small amount of water then autoclaved at 15psi at 121°C for 15 minutes, then depressurized and cooled for 30 minutes. After cooling, the

sterilized contents were poured onto a sterile brown paper towel laid over a dish (working under a fume hood) to filter the shards from the water. The brown paper towel proved to be the easiest surface for removal of the CNF and allowed easy transfer to soak in a solution of 95% ethanol while remaining on the paper towel. The microscalpels and other instruments were also sterilized in a solution of 95% ethanol, then left to dry under the fume hood along with the implants prior to the surgery. The microscalpels and forceps were also sterilized in 95% ethanol for 30 seconds between each fish procedure.

The implantation setup was assembled and the agar plate was put in place (*Figures 12.2, 12.4*). Before being positioned on the agar mount, each fish was immersed in a solution of 200 mg/liter Tris-buffered tricaine methanesulfonate in deionized water to ensure proper anesthetization. Following anesthesia, the procedure described above was executed along the dorsal line caudal to the dorsal fin. The incision was intramuscular only, rendering the implant completely subcutaneous for three-dimensional cellular integration.

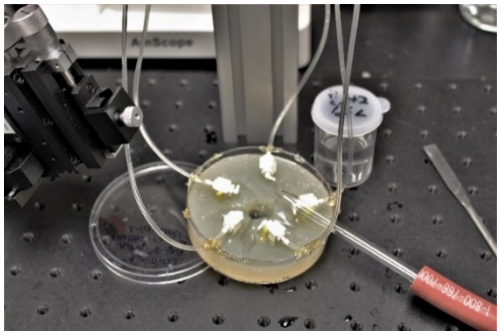


Figure 12.2. Agar plate/water supply apparatus

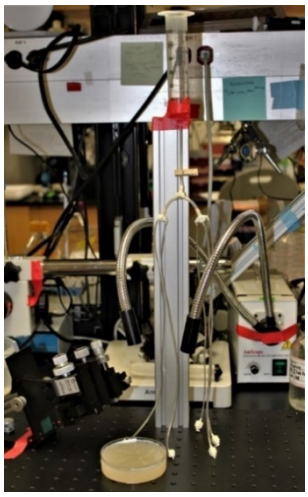


Figure 12.4. Total operation setup

Postsurgical Monitoring

Immediately upon completion of the surgery, stress experienced by the fish was managed by placing the fish in a tank of egg water to regain consciousness, then gently reintroducing the fish to their holding tank to allow acclimatization. The holding tank contained a solution of cephalexin (6.6mg/L) and methylene blue (30mg/L) to prevent bacterial and fungal infection, respectively, that could interfere with, or alter, the response to the implant. The zebrafish were maintained in this antibiotic solution for 3 days post-operation following the protocol by Fang et. al (32) (Appendix B.6), then returned to normal holding conditions. The fish were kept in the same tank setup as the PTU treatment, utilizing the mesh baskets for the daily solution change because the flow-through system was unsuitable for antibiotic immersion. The negative effects encountered from the PTU treatment were not expected to occur due to the shorter duration of containment and lack of food buildup, as the fish were too stressed to eat. This was evident from a preliminary attempt to introduce food on the second day of antibiotic treatment, eliciting a disinterested response from the fish.

The physical movement of the fish was observed post-operation for at least 10 minutes to ensure that normal behavior was restored. If severe distress was evident from unusual behavior (i.e. prolonged erratic swimming, persistent color change indicating stress, etc.), the affected fish was removed from the experiment and promptly euthanized using an overdose of sodium bicarbonate- buffered MS222 (300 mg/L) for a duration of 10 minutes or until the heart had stopped beating to avoid extended distress. Since the surgical procedure was performed only once during the initial portion of the experiment, to prevent

deleterious side-effects such as weakened cardiovascular strength, no other analgesics were administered after the zebrafish had regained consciousness.

Imaging

Post-surgery brightfield and confocal fluorescence microscopy was conducted on the zebrafish using an inverted Olympus IX-81 motorized microscope (33), in conjunction with an Olympus FLUOVIEW FV1000 confocal scanning unit and image acquisition software. To contain the zebrafish and minimize variation of positioning between fish, a fish-shaped barrier was created using waxed yarn (Wikki Stix®, (34)) that adhered to the temperature-controlled ITO-coated Delta T-dishes, while allowing water to flow into the resulting enclosure (*Figure 13.1*). The Delta T-dishes were secured in a thermo-stage that was used to provide support when placed on the microscope (*Figure 13.2*). After 3 days post-operation, the zebrafish were immobilized with MS-222 (200 mg/liter Tris-buffered tricaine methanesulfonate), then collected with a plastic transfer pipet, trimmed to accommodate the size of the fish, and laid into the corralled region on the Delta T-dish.

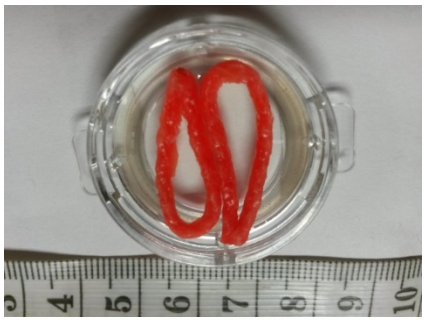


Figure 13.1. Delta T-dish (0.5mm) with waxed yarn barriers (cm)

Figure 13.2. Delta T-dish on stage adapter for fish (cm)

The duration of the imaging procedure was dependent on the number of layers that were scanned. The MPO Casper crosses required a step size of no more than 2.00µm

between scan layers for detection of neutrophil cells, taking up to 8 minutes to complete a total scan, while the fli1 Casper crosses were imaged with a step size of 5.00μm, taking up to 5 minutes (refer to Appendix A.13 for scan settings). The solution in the Delta T-dish was changed frequently with a pipet while on the microscope stage. The MS-222 solution was replaced with system water near the end of imaging to prevent prolonged exposure, an event that seemed to result in death in imaged fish.

III. RESULTS

Zebrafish Lines Used

The original intent of this experiment was to cross Casper *Danio rerio* with MPO and fli1 lines to produce transparent juveniles containing the fluorescence properties described above. The crossbreeding was performed in the zebrafish facility at the University of Maine by the facility manager. At 1.5 wpf it was observed that pigmentation was a dominant trait in several crossed individuals from each transgenic line and stripes were apparent in the fish. For this reason, administration of PTU was initiated on separate groups of MPO and fli1 *Danio rerio* to remove pigmentation while preserving EGFP expression in the neutrophils and vasculature, respectively, of the zebrafish. The Casper crosses continued to develop and were raised separately alongside the treated transgenic lines in the secondary zebrafish facility, also at the University of Maine.

Eventually, it became clear that the PTU-treated MPO and fli1 fish were smaller in size and number than required for the experiment. The drastic population decline coupled with the time constraint of the project led to the termination of the PTU-treated groups. The Casper crosses were maintained and utilized for the implantation surgery despite visible pigmentation along the zebrafish body (*Figures 14.1-14.2*).

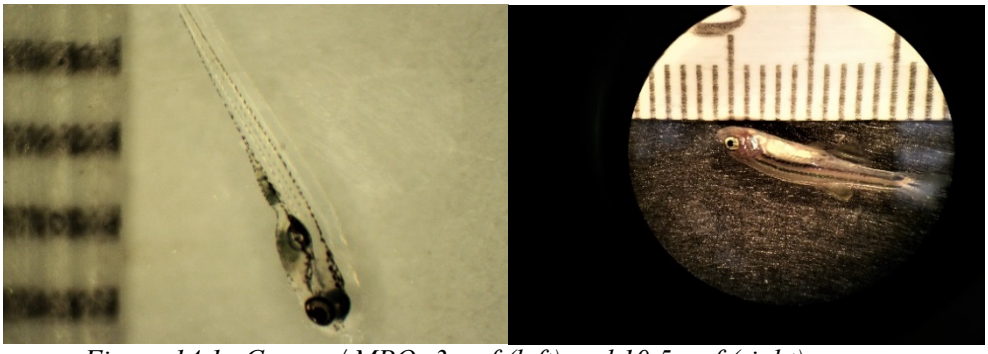


Figure 14.1. Casper / MPO: 3 wpf (left) and 10.5 wpf (right)

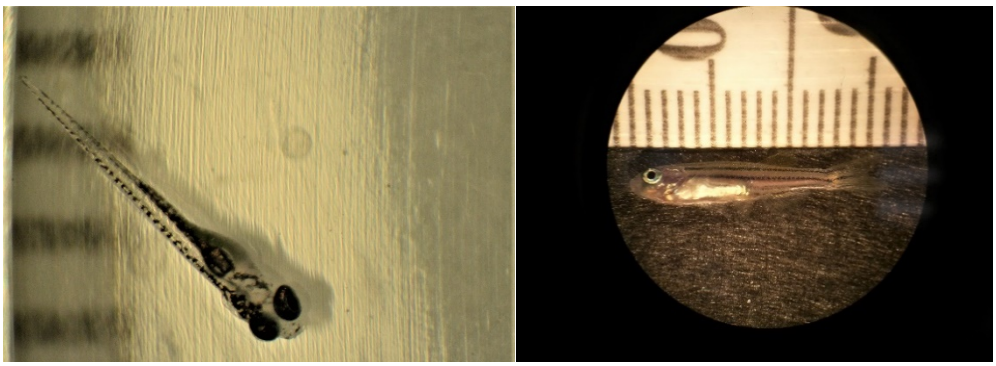


Figure 14.2. Casper / fli1: 3 wpf (left) and 10.5 wpf (right)

Performing the Surgery

The main procedure was more arduous than the preceding tests due to the fact that the MPO and fli1 Casper crosses were less developed than the 5 month-old fish used for practice surgeries. At only 7 wpf, and with mixed sizes ranging from 8 to 16mm, it was challenging to follow the same implantation protocol on each fish. Furthermore, the glassshard trial did not work because it was impossible to ensure that the clear material had been placed sub-epidermally, and the shard was also difficult to manipulate with the 15° microscalpel. The use of forceps was also attempted with the same level of success as the microscalpel, and the glass trial was discarded. The length of the procedure was dependent on the trial: Incision-only lasted about 30 seconds per fish, and CNF implantation required up to 2 minutes per fish to complete. Incision size was refined to minimize affected tissue and reduce the potential of residual pain by using the technique described for the practice tests. No sealant was used on the incision post-implantation as zebrafish skin completely re-epithelializes within 24 hours (15), reducing the chance of environmental contamination.

The water supply apparatus worked as predicted; however the length of the procedure varied depending on the size of the individual fish, so the intention to complete

five zebrafish operations in succession was unreasonable and the multiple tubes were unnecessary. The impressions in the agar were also slightly oversized for the smaller zebrafish, and though an assistant used vacuum aspiration to help remove excess water, slippage of the fish was still occurring and the microscalpel blade was deflected as before. A plain poured agar plate and water dripped on the fish with a plastic pipet was implemented instead of the large apparatus. This procedure was completed as quickly as possible to prevent suffocation of the fish, and the water was applied with a controlled effort to avoid the implantation site to minimize blade deflection on the epidermis.

Post-surgery Survival

On average, the fli1-Casper fish were slightly larger than the MPO-Casper fish at the time of the surgery. It was hypothesized that this would improve chances of survival, however this was not the case, perhaps because the fli1 fish were operated on first and the technique was not as consistent. The survival of each trial of zebrafish was assessed 24 hours after the operation was performed and continuously through the imaging process. The day following the procedure resulted in the highest fatalities (*Figure 15*), specifically in the CNF trials. No fish died during the last two days of the post-surgical treatment with cephalexin and methylene blue. Once imaging was initiated, it was observed that fatalities would occur inconsistently between trials based on the stamina of individual fish during the imaging process. After the procedure, the additional exposure to the MS-222 tricaine sedative solution often resulted in zebrafish death, which may have indicated that the injured fish had developed an intolerance for the MS-222. It is also possible that the fish

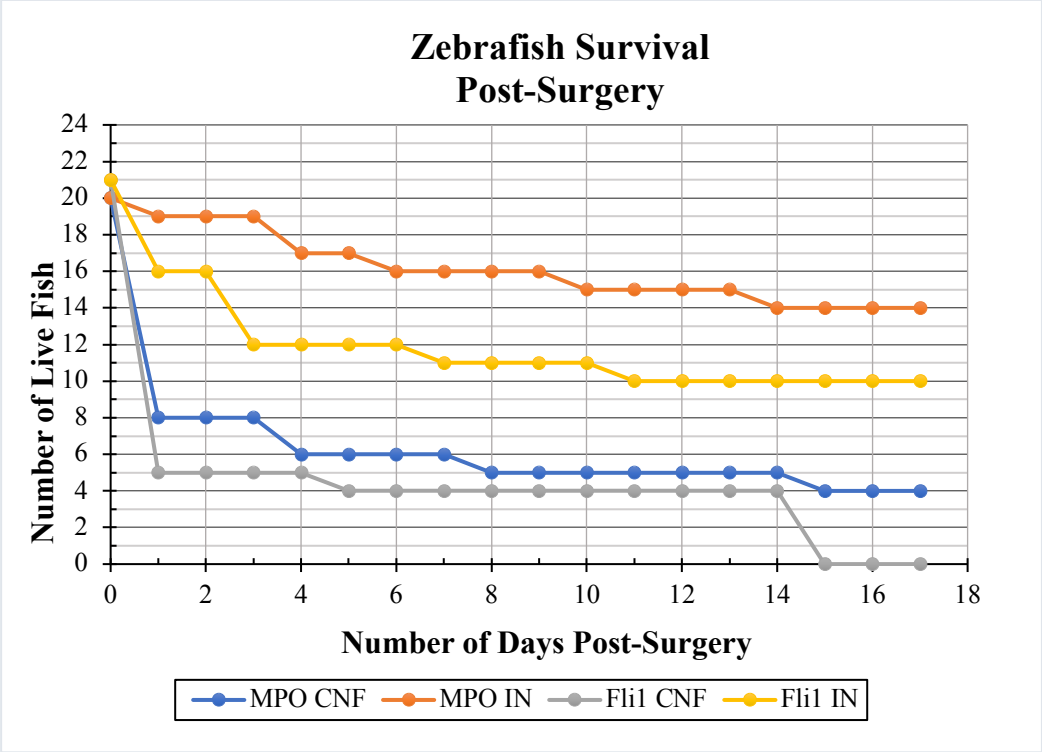


Figure 15. Post-surgery survival of Casper crosses by transgenic type and surgical trial. Key: 'MPO' denotes MPO-Casper transgenic fish, and 'Fli1' denotes fli1-Casper transgenic fish. 'IN' indicates incision-only trials, and 'CNF' indicates the CNF shard trials.

were not adequately oxygenated during the longer image scans and succumbed to the oxygen deficiency.

These results prompted changes in the procedure to improve survival: The fish were left in the MS-222 tricaine solution just until they were anesthetized, then quickly transferred to the prepared Delta T-dish with more MS-222 solution dripped over their gills, eventually filling the dish. The solution was removed from the dish and replaced with system water after each scan and during mid-imaging positioning to increase oxygenation. Zebrafish fatalities decreased after these changes were made during imaging, however there was a sharp decline in the population after several fish managed to escape their tanks through the flow-through outlet into the pipe leading to the filter through which all system

water passed. Despite the plastic guards that were placed in the tanks to prevent this from happening, a few fish originating from different trials were discovered on the filter, killed from suffocation. Thirteen days after the operation, all remaining fli1-Casper CNF zebrafish were found to have disappeared from their tank, though there were no fish on the filter. The logical explanation was that they had escaped in the manner described above, then the filter was cleaned overnight, removing evidence of the event. Imaging ceased shortly after for all trials, and the remaining fish thrived with continued maintenance until they were euthanized according to the standard protocol (Appendix D) upon conclusion of the experiment.

Postsurgical Trends in fli1-Casper Zebrafish

As previously stated, the fli1-Casper trials suffered substantial post-surgery fatalities, leading to a poor yield of viable data points. In the fli1-Casper incision (fli1-IN) trial, 16 out of 21 fish survived, and only 5 out of 21 fish survived in the fli1-Casper CNF (fli1-CNF) trial. In general, the fli-1 Casper zebrafish did not present clear expression of vasculature to the same extent as the control fli1 zebrafish (*Figures 16.1, 16.8*), hindering assessment of the resulting images from the operation trials. This could have occurred from an unanticipated error in the genetic crossing of the fli1 and Casper fish, already evident from the presence of pigmentation when the crossed zebrafish should have been transparent; it is also possible the crossbred zebrafish may have been hemizygous.

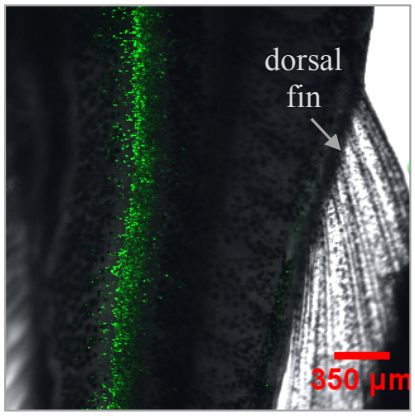


Figure 16.1. EGFP on vasculature, control fish

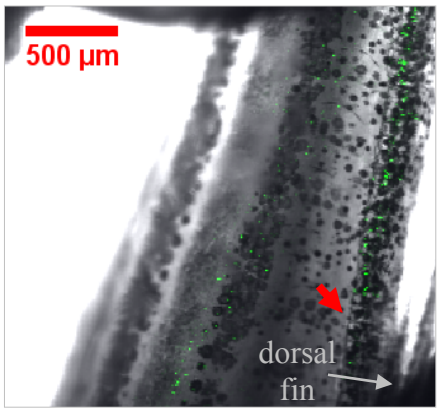
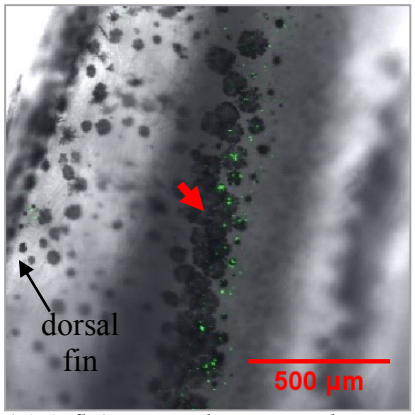


Figure 16.8. fli1-IN: Red arrow indicates re-epithelialized incision at 9 dps

Imaging of the fli1-IN trial illustrated a lack of expression, indicating additional vascularization had occurred (*Figures 16.6, 16.10)*, however disruption of existing vasculature was evident in some fish where the incision had crossed the vertebral arteries (*Figure 16.9*). The injuries in this trial re-epithelialized rapidly and localization of endothelial cells around the wound diminished with time, suggesting a decline in their recruitment to the incision site during the completion of the healing process.



16.6. fli1-IN: Red arrow indicates some concentration of endothelial cells, no CNF retention; 7dps

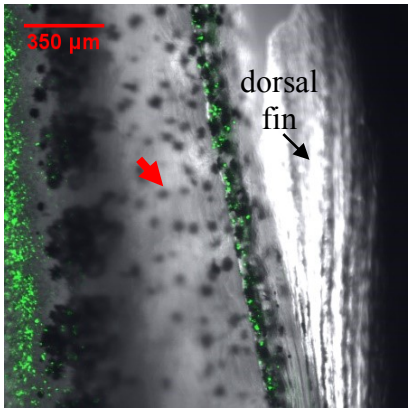


Figure 16.10. fli1-IN: Red arrow indicates high concentration of endothelial cells along vasculature, re-epithelialized incision; 15 dps

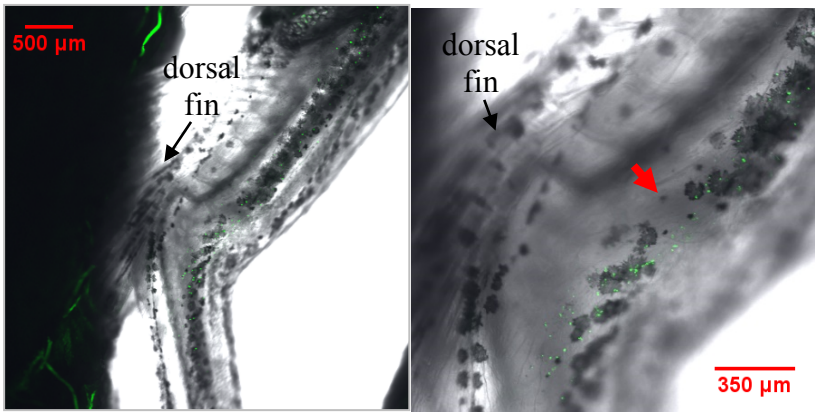


Figure 16.9. fli1-IN:Red arrow indicates endothelial cells viewed along vasculature, re-epithelialized injury with malformed tail; 15dps

There was no retention of CNF in the fli1-CNF trial, rendering the affected fish as identical to the fli1-IN trial. Most fish from the fli1-CNF trial possessed an empty pocket where the CNF had been implanted, and some endothelial cells were evident in the area around this region, but definitive vascularization had not developed. Due to the lack of CNF, this trial presented no obvious indication of vascularization in the material, therefore examination of this portion of the experiment could progress no further. The sudden population decline from the escaped fish also contributed to the closure of the fli1-Casper trials as there were insufficient numbers of fish to provide a comparison between trials, which at that point had consisted of injury (fli1-IN) and even greater injury (fli1-CNF).

Postsurgical Trends in MPO-Casper Zebrafish

Though the MPO-Casper trials sustained fewer fatalities than the fli1-Casper trials, the number of fish in the MPO-Casper CNF (MPO-CNF) trial was much fewer than required to allow determination of statistical significance. From a population of 20 fish, only eight survived after surgery, while 19 survived in the MPO-Casper incision (MPO-IN) trial. In addition, few fish retained the implant in the MPO-CNF trial which further

reduced the probability of detecting trends in neutrophil migration. Despite the lack of statistically relevant data, general observations could be made between the incision and implant trials which could help to guide future experiments.

Heightened concentration of neutrophils was displayed around the region of interest in the MPO-IN trial as compared to the same region in the MPO control trial, indicating that neutrophil migration was prompted by inflicting an injury (*Figures 17.2, 17b.a*). A distinct increase in the density of neutrophils occurred around the incision site in several fish *(Figures 17a-17e)*. It was also observed that the incision had re-epithelialized almost completely in most of the imaged fish, and the site of injury was identified by a slight absence of pigment. As imaging did not begin until three days post-operation, the occurrence of re-epithelialization during the treatment period was not surprising. There appeared to be greater localization of an immune response around the implant site in the MPO-CNF trial than in the MPO-IN trial based on a greater presence of bright spots, indicating neutrophils. For the fish that did not retain the implant, this could have been due to a larger injury being created during the implantation. More practice performing the implantation procedure would afford more precision and would help refine the injury area. These fish also displayed predominantly re-epithelialized injuries.

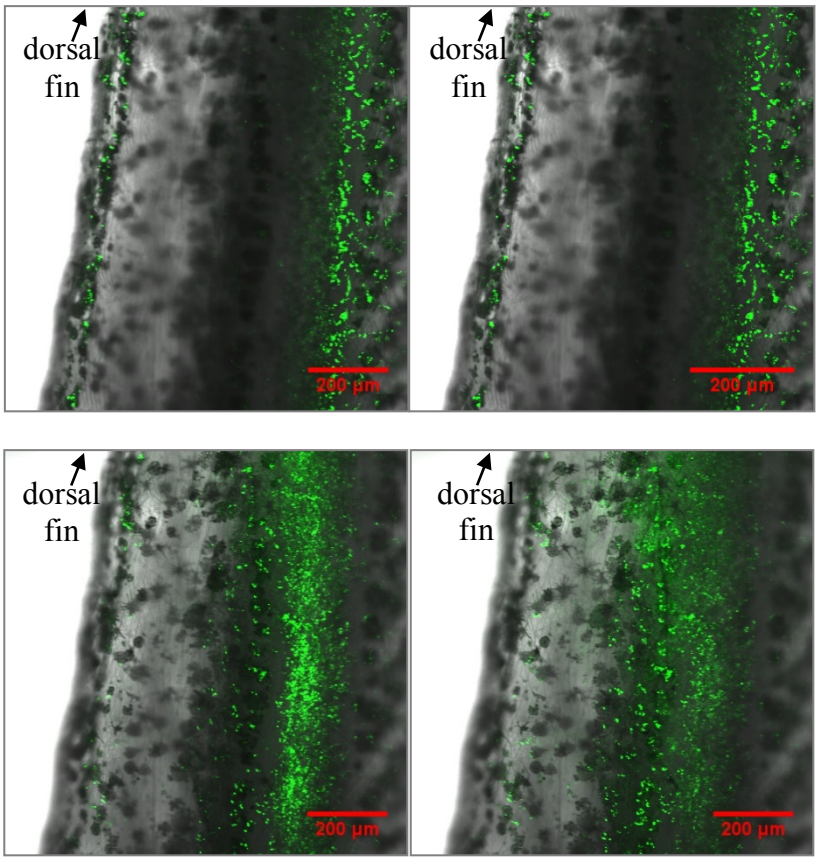


Figure 17.2. Control MPO: Concentration of neutrophils shown across multiple confocal image layers

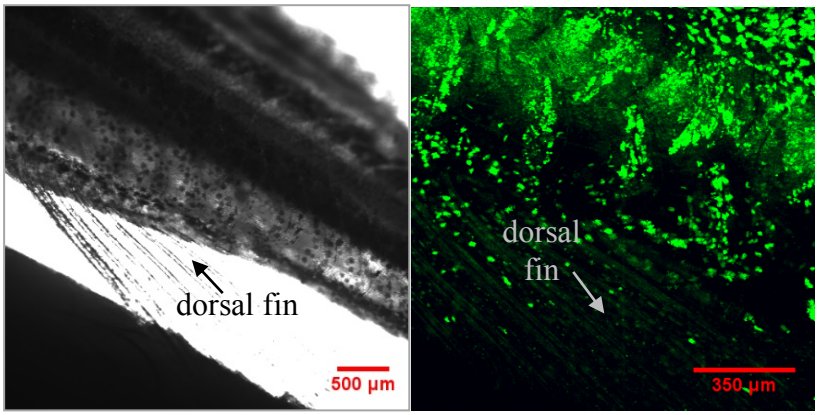


Figure 17.3. MPO-IN: Neutrophil concentration around site of incision, 4dps

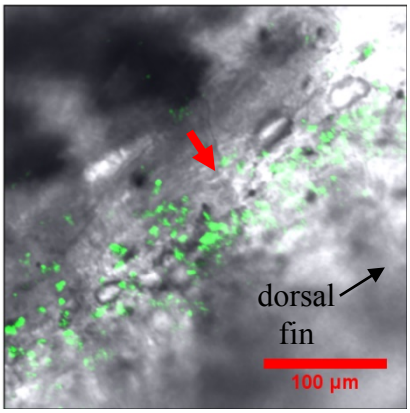


Figure 17.4. MPO-CNF: Red arrow indicates neutrophil concentration around implantation site; no CNF retention, 4dps

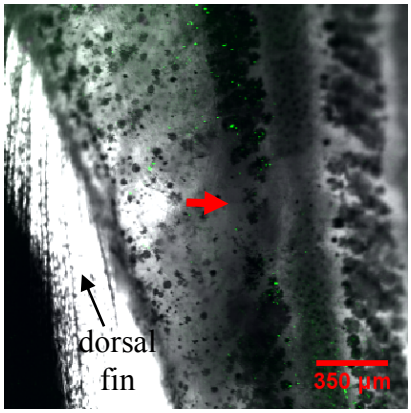


Figure 17.5. MPO-IN: Red arrow indicates pigment interruption, neutrophil concentration around the incision site, 8dps

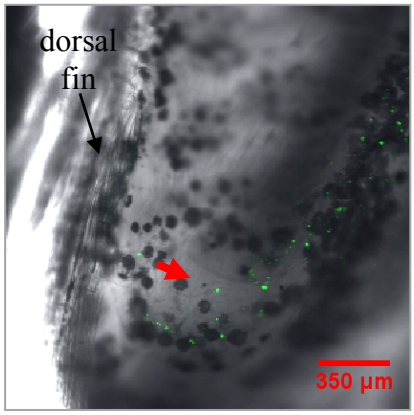


Figure 17.9. MPO-IN: Red arrow indicates concentration of neutrophils around site, 8dps

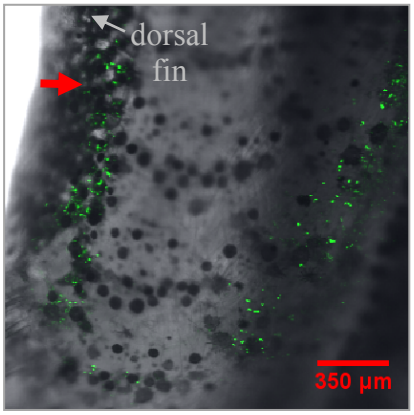


Figure 17.10. MPO-IN: Red arrow indicates greater neutrophil concentration in confocal layer superior to 17.9

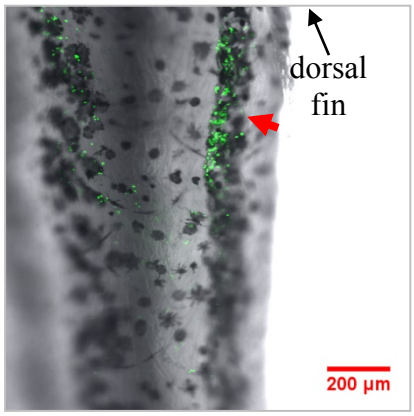


Figure 17.15. MPO-IN: Red arrow indicates re-epithelialized incision and some neutrophil concentration remaining along incision site, 14dps

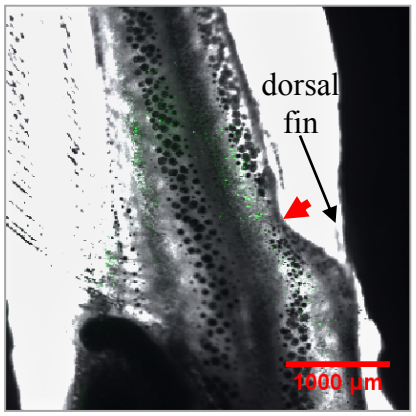


Figure 17.13. MPO-CNF: Red arrow points to pocket indicating lack of CNF retention, surrounding neutrophil concentration apparent

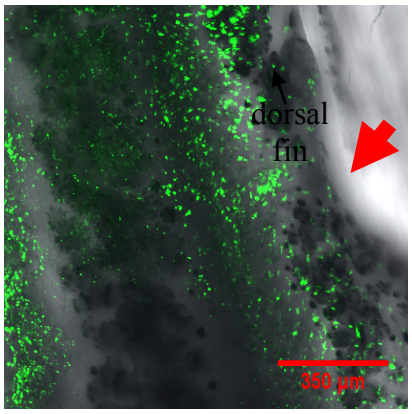


Figure 17.14. MPO-CNF: Close-up of 17.13; neutrophil concentration is more visible

As previously stated, few zebrafish retained the CNF implant, most likely due to improper establishment of the shard during surgery. However, three MPO-CNF zebrafish exhibited a small dark spot where the implant had been inserted (*Figures 17.6-17.8)*. The CNF shards were difficult to discern from the surrounding tissue upon magnification and EGFP was not visible, lending inconclusive evidence of a direct neutrophil response to the implant.

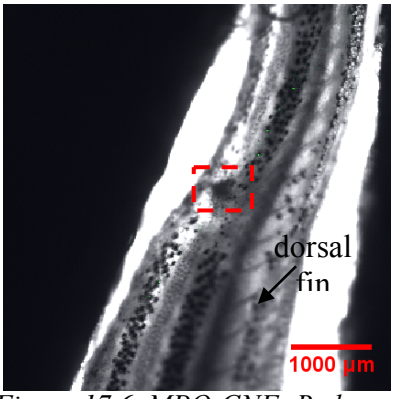


Figure 17.6. MPO-CNF: Red box indicates retained CNF implant, 6dps

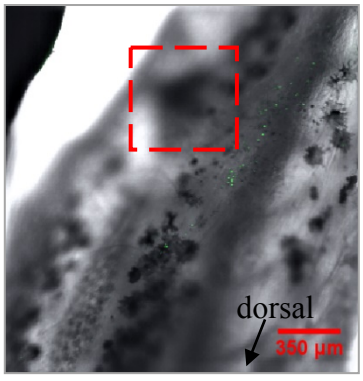


Figure 17.7. MPO-CNF: Red box indicates retained CNF, surrounding neutrophil is present

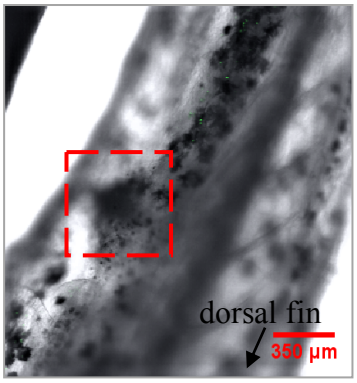


Figure 17.8. MPO-CNF: Red box indicates retained CNF, neutrophil concentration in same plane as CNF implant, determined by confocal imaging

IV. DISCUSSION

This project encompassed an array of experiments directed toward the development of a novel implantation procedure and involved the design and execution of supplemental surgical tools and methods protocols, providing the initial advances of this pilot study. It can be concluded that it is achievable to insert CNF shards sub-epidermally in the adult zebrafish model and to monitor both implant retention and fish survival while evaluating neutrophil migration or vasculature organization using confocal fluorescence imaging in pigmented juvenile zebrafish. Furthermore, zebrafish immersion protocols have been developed, involving fabrication of a nylon mesh basket to permit exposure to solutes in the suspending medium. Construction of a reverse-mold and a water supply apparatus was accomplished to support zebrafish survival during surgery,

Numerous potential applications for nanocellulose have been identified and many have yet to be investigated. Extension of this pilot project will provide greater insight into the biocompatibility of CNF and evaluate its potential as a suitable implant material. Whether for use in humans or as a biodegradable fish tagging device, the developed implant could contribute to existing technology. Although this study did not to provide conclusive evidence regarding immune response and potential for vascularization of CNF shards in zebrafish, it serves as a preliminary effort on which to base future studies in the application of nanocellulose materials in living systems.

V. RECOMMENDATIONS FOR FUTURE WORK

Future observation of the implantation of CNF would benefit from utilizing 4-month or older zebrafish of homogeneous size to increase the chance of subdermal implant establishment, as well as fish survival. The technique using the micromanipulator and microscalpels can be improved through extensive practice to increase uniformity of the injuries between individual fish. To further promote post-surgery survival, the water supply apparatus and reverse-mold agar plate should be modified to accommodate both the dimensions of the fish and the number of fish that are arranged on the agar and ready for operation. The largest issue during surgery was water on the epidermis of the fish deflecting the blade of the microscalpel. A method to isolate the operation site could be developed, possibly a barricade between the dorsal fin and the gills to direct the water for precise oxygenation without compromising the surgery.

The imaging procedure would benefit from selecting transgenic zebrafish that undergo successful crossbreeding, where the Casper line crossed with the MPO and fli1 lines yields transparent fish instead of those with pigment, as in this experiment. Alternatively, a protocol for long-term PTU administration could be developed, accounting for chemical concentration, fish density, and waste removal during maintenance. Utilization of more powerful image acquisition and processing software would permit a more thorough observation of the vascular and neutrophil behavior in the zebrafish upon introduction of CNF, as well as the examination of CNF surface interactions with zebrafish tissues and interstitial fluid. Furthermore, a lower long-term dosage of MS-222 tricaine solution may prove necessary to prevent fish fatalities during the imaging process.

LIST OF REFERENCES

1. Arizona Game and Fish Department. Fish Tagging and Marking Techniques [Internet]. 2013 [cited 2017 May 9]. Available from: http://www.azgfd.gov/w_c/Fish_Tagging_Marking_Techniques.shtml/

2. Sciences B. PIT Tagging : Simple Technology at Its Best. 2017;54(5):447–54.

3. Baras E, Malbrouck C, Houbart M, Kestemont P, Melard C. The effect of PIT tags on growth and physiology of age-0 cultured Eurasian perch Perca flu Õ iatilis of variable size. 2000;

4. Rompen E. The effect of material characteristics , of surface topography and of implant components and connections on soft tissue integration : a literature review. 2006;17:55–67.

5. Bo O, Pihlajama H. Clinical biocompatibility of biodegradable orthopaedic implants for internal " xation : a review. 2000;21.

6. Ambrose CG, Clanton TO. Bioabsorbable Implants : Review of Clinical Experience in Orthopedic Surgery. Ann Biomed Eng. 2006;32(1):171–7.

7. Jorfi M, Foster EJ. Recent advances in nanocellulose for biomedical applications. J Appl Polym Sci. 2015;132(14).

8. Morán JI, Alvarez VA, Cyras VP, Vázquez A. Extraction of cellulose and preparation of nanocellulose from sisal fibers. Cellulose. 2008;15(1):149–59.

9. Lin N, Dufresne A. Nanocellulose in biomedicine: Current status and future prospect. Eur Polym J [Internet]. 2014;59:302–25. Available from: http://www.sciencedirect.com/science/article/pii/S0014305714002493

10. Torres FG, Troncoso OP, Torres C, Grande CJ. Advances in Natural Polymers. Adv Struct Mater [Internet]. 2013;18:21–54. Available from: http://www.scopus.com/inward/record.url?eid=2-s2.0-84874283870&partnerID=tZOtx3y1

11. Abraham E, Deepa B, Pothan LA, Jacob M, Thomas S, Cvelbar U, et al. Extraction of nanocellulose fibrils from lignocellulosic fibres: A novel approach. Carbohydr Polym [Internet]. 2011;86(4):1468–75. Available from: http://dx.doi.org/10.1016/j.carbpol.2011.06.034

12. Prakash P, Kasaby HE, Aggarwal RK, Humfrey S. Microincision bimanual phacoemulsification and Thinoptx implantation through a 1.70 mm incision. Eye (Lond). 2007;21(2):177–82.

13. Fine IH, Hoffman RS, Packer M. Optimizing refractive lens exchange with bimanual microincision phacoemulsification. J Cataract Refract Surg. 2004;30(3):550–4.

14. Ahoor M, Sorkhabi R. Intraocular Foreign Body Removal by 23-Gauge Micro Incision Vitrectomy Surgery and Back Flush Flute Needle: A Case Series Study. Glob J Health Sci [Internet]. 2016;9(5):262. Available from: http://www.ccsenet.org/journal/index.php/gjhs/article/view/56079

15. Sambasivarao S V. OCT-Documented incision features and natural history of clear corneal incisions used for bimanual microincision cataract surgery. Cornea. 2013;18(9):1199–216.

16. Gerlach GF, Morales EE, Wingert RA. Microbead Implantation in the Zebrafish Embryo. J Vis Exp [Internet]. 2015;(101):e52943. Available from: http://www.jove.com/video/52943/microbead-implantation-in-the-zebrafish-embryo

17. Brittijn SA, Duivesteijn SJ, Belmamoune M, Bertens LFM, Bitter W, De Bruijn JD, et al. Zebrafish development and regeneration: New tools for biomedical research. Int J Dev Biol. 2009;53(5–6):835–50.

18. Richardson R, Slanchev K, Kraus C, Knyphausen P, Eming S, Hammerschmidt M. Adult zebrafish as a model system for cutaneous wound-healing research. J Invest Dermatol [Internet]. 2013;133(6):1655–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23325040%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3644348

19. Chang W, Hwang P. Development of Zebrafish Epidermis. 2011;214(Part C):205–14.

20. Sullivan C, Kim CH. Zebrafish as a model for infectious disease and immune function. Fish Shellfish Immunol. 2008;25(4):341–50.

21. Lambris JD. Current Topics in Innate Immunity II.

22. Mathias JR, Perrin BJ, Liu T, Kanki J, Look AT, Huttenlocher A. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish flammation is a critical process during normal im-. 2006;80(December).

23. Rosales C, Demaurex N, Lowell CA, Uribe-querol E. Neutrophils : Their Role in Innate and Adaptive Immunity. 2016;2016:2–4.

24. Developmental stage data for this paper were retrieved from the Zebrafish Information Network (ZFIN); University of Oregon; Eugene; OR 97403-5274; URL: http://zfin.org/; 9/20/17.

25. Renshaw S, Loynes C. A transgenic zebrafish model of neutrophilic inflammation.

Blood… [Internet]. 2006;108(13):3976–8. Available from: http://bloodjournal.hematologylibrary.org/content/108/13/3976.short

26. Lawson ND, Weinstein BM. In Vivo Imaging of Embryonic Vascular Development Using Transgenic Zebrafish. Dev Biol [Internet]. 2002;248(2):307–18. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0012160602907116

27. White RM, Sessa A, Burke C, Bowman T, Leblanc J, Ceol C, et al. Resource Transparent Adult Zebrafish as a Tool for In Vivo Transplantation Analysis. 2008;(February):183–9.

28. Karlsson J, von Hofsten J, Olsson P-E. Generating Transparent Zebrafish: A Refined Method to Improve Detection of Gene Expression During Embryonic Development. Mar Biotechnol [Internet]. 2001;3(6):0522–7.

29. Tobias CW, Eisenberg M. IN ELECTROLYSIS-- REVIEW. 99(12):359–65.

30. Moore SW, Kennedy TE. Dissection and culture of embryonic spinal commissural neurons. Curr Protoc Neurosci. 2008;(SUPPL. 43).

31. Conrad GW, Bee JA, Roche SM, Teillet MA. Fabrication of microscalpels by electrolysis of tungsten wire in a meniscus. J Neurosci Methods. 1993;50(1):123–7.

32. Fang P, Lin JF, Pan HC, Shen YQ, Schachner M. A Surgery Protocol for Adult Zebrafish Spinal Cord Injury. J Genet Genomics [Internet]. 2012;39(9):481–7.

33. OLYMPUS. Research Inverted System Microscope Ix71/Ix81 Ix2 Series. In: Manual.

34. Wikki Stix One of a Kind Creatables [Internet]. Available from: https://www.wikkistix.com/

35. Hachicho N, Reithel S, Miltner A, Heipieper HJ, Küster E, Luckenbach T. Body mass parameters, lipid profiles and protein contents of zebrafish embryos and effects of 2,4-dinitrophenol exposure. PLoS One. 2015;10(8):1–19.

36. Avella MA, Place A, Du SJ, Williams E, Silvi S, Zohar Y, et al. Lactobacillus rhamnosus Accelerates Zebrafish Backbone Calcification and Gonadal Differentiation through Effects on the GnRH and IGF Systems. PLoS One. 2012;7(9).

37. Hohn C, Petrie-Hanson L. Evaluation of visible implant elastomer tags in zebrafish (Danio rerio). Biol Open [Internet]. 2013;2(12):1397–401.

38. Peng S, Yao L, Cui C, Zhao H, Liu C, Li Y, et al. Semaphorin4D promotes axon regrowth and swimming ability during recovery following zebrafish spinal cord injury. Neuroscience [Internet]. 2017;351:36–46.

39. Collymore C, Tolwani A, Lieggi C, Rasmussen S. Efficacy and safety of 5 anesthetics in adult zebrafish (Danio rerio). J Am Assoc Lab Anim Sci. 2014;53(2):198–203.

40. Huang W-C, Hsieh Y-S, Chen I-H, Wang C-H, Chang H-W, Yang C-C, et al. Combined Use of MS-222 (Tricaine) and Isoflurane Extends Anesthesia Time and Minimizes Cardiac Rhythm Side Effects in Adult Zebrafish. Zebrafish [Internet]. 2010;7(3):297–304.

APPENDICES

APPENDIX A: FIGURES

APPENDIX A.1:

Block of dehydrated CNF from which the implants were shaved (cm/mm)

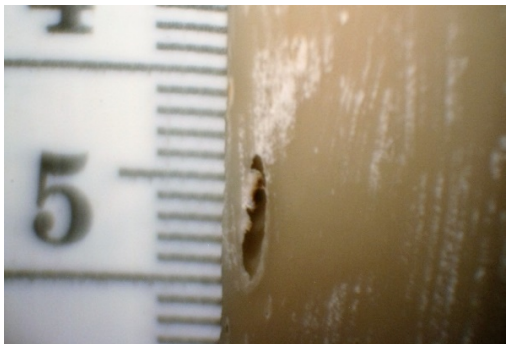


Figure 1.1. Air bubble in block

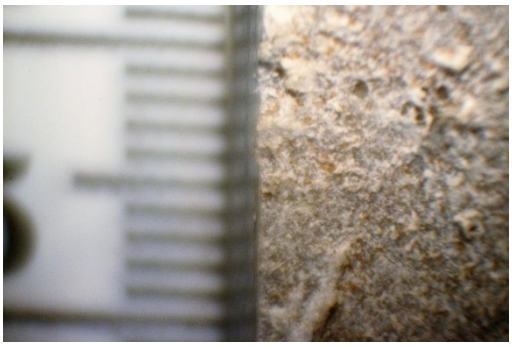


Figure 1.2. Detail of fiber formation

Figure 1.3. View of block after squaring

APPENDIX A.2:

Shaving the CNF

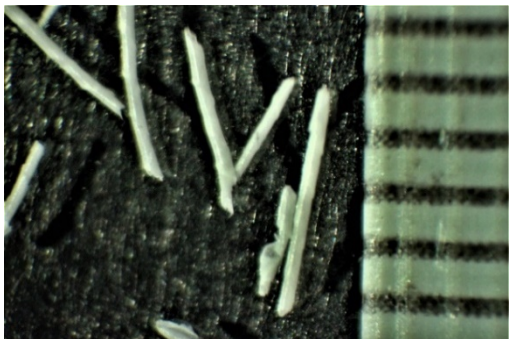


Figure 2.1. Spindles generated with end mill (~0.05mm thickness)

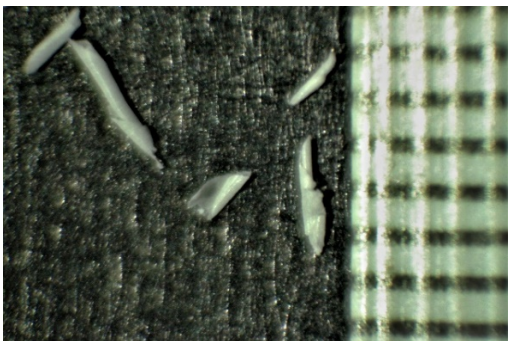


Figure 2.2. Spindles generated with end mill (~0.025mm thickness)

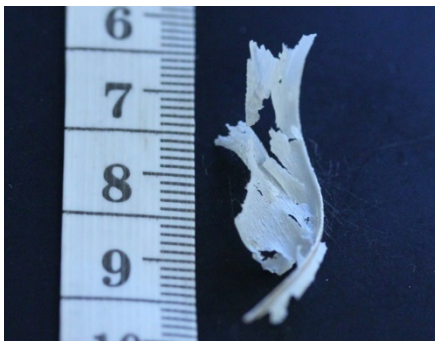


Figure 2.3. Shavings generated with hand plane (>0.05mm thickness)

Figure 2.4. Shavings generated with hand plane (<0.05mm) thickness)

APPENDIX A.3:

Shaping the CNF

MODIFICATIONS (mm):

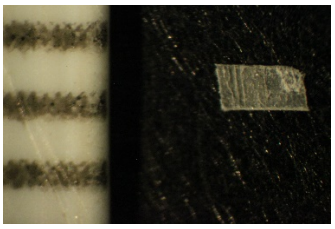


Figure 3.1. CNF in rectangle form

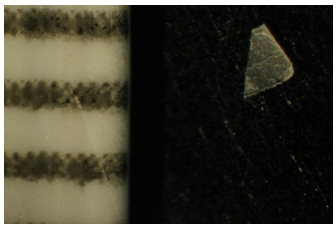


Figure 3.2. CNF in triangle form

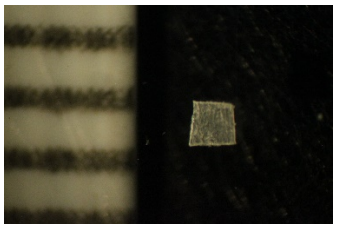


Figure 3.3. CNF in square form

SHAPING SETUP (cm/mm):

Figure 3.4. Angled utility knife

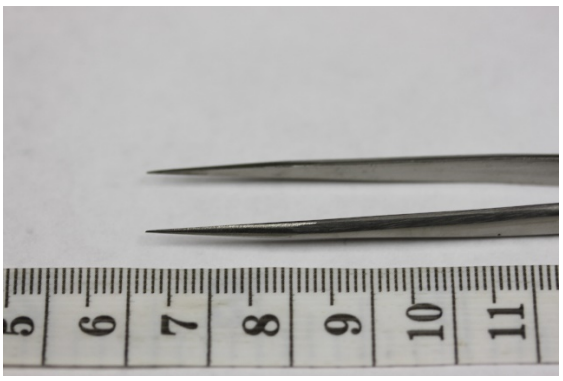


Figure 3.5. Forceps

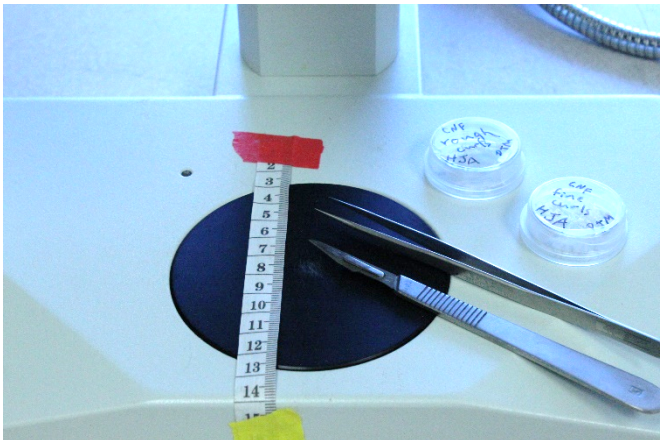


Figure 3.6. Complete shaping setup

APPENDIX A.4:

CNF swelling (mm)

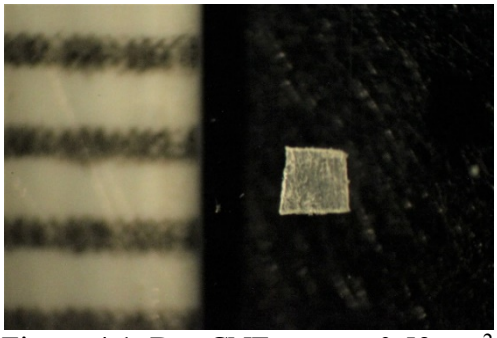


Figure 4.1. Dry CNF square, 0.53mm^2

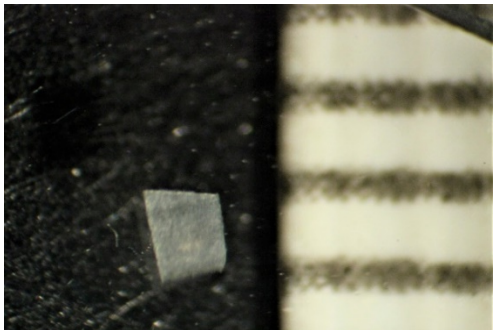


Figure 4.2. Soaked for 22 hours in deionized water, 0.68mm^2 (22% increase in area)

APPENDIX A.5:

Transgenic zebrafish lines

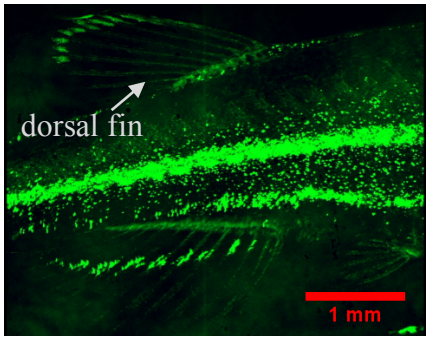


Figure 5.1. fli1 *Danio rerio*, 4x objective

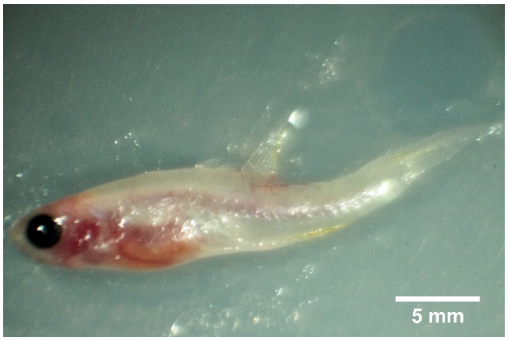


Figure 5.2. Euthanized Casper *Danio rerio,* 5 months old

APPENDIX A.6:

Zebrafish salvaged from PTU treatment - 11 wpf

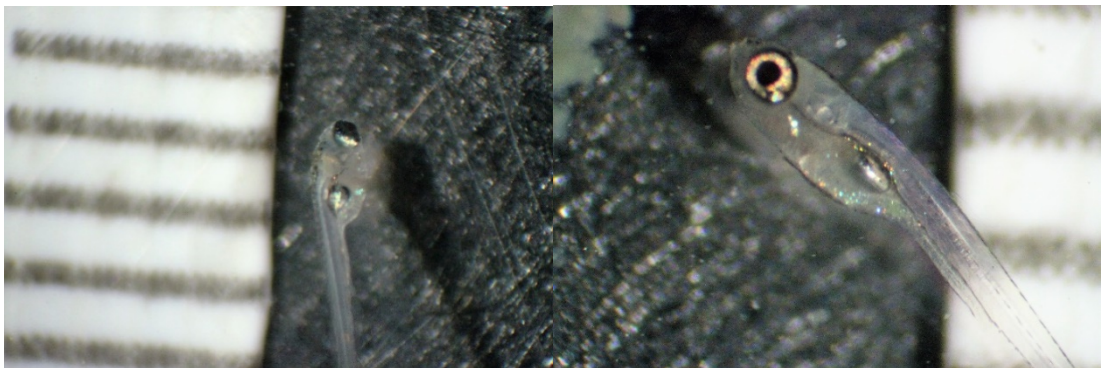


Figure 6.1. Fli1 *Danio rerio* (mm)

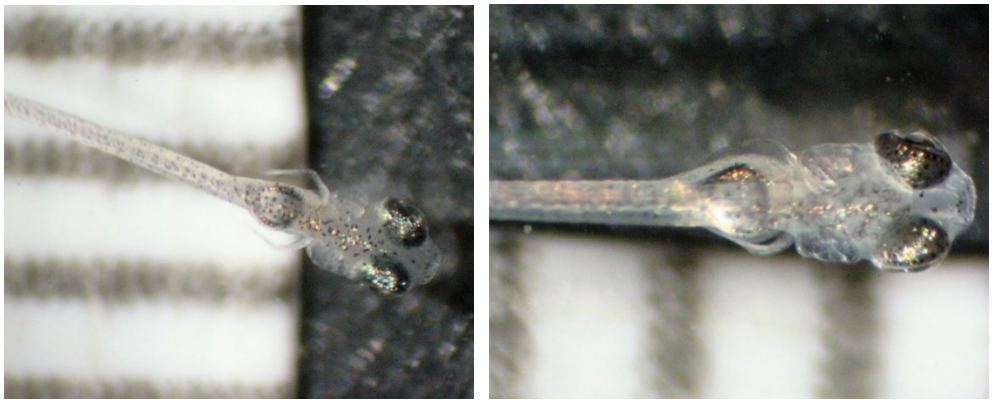


Figure 6.2. MPO *Danio rerio* (mm)

APPENDIX A.7:

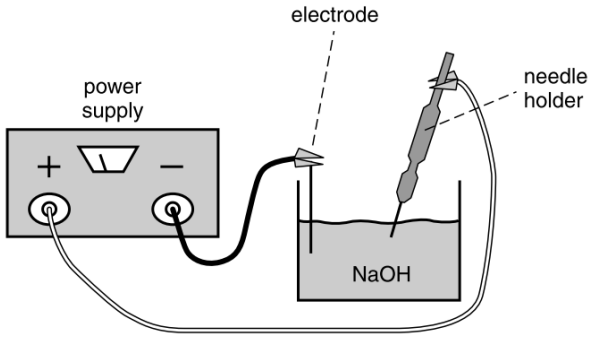


Figure 7.1. Electrolysis setup. Reproduced from Ref [30].

Figure 7.2. Vertical submersion

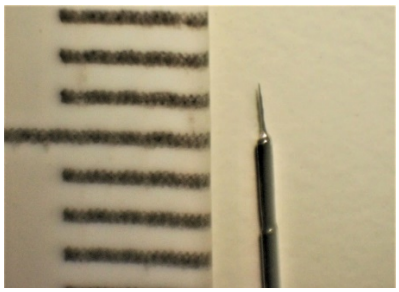


Figure 7.3. Vertical submersion, gradual withdrawal

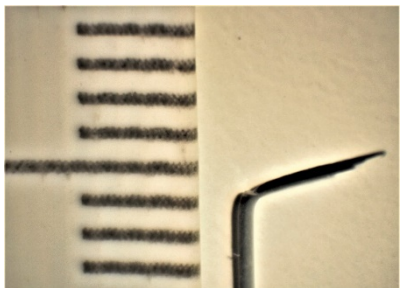


Figure 7.4. Meniscus, flat edge

Figure 7.5. Meniscus/vertical submersion, hook

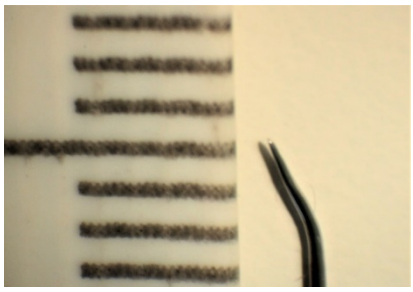


Figure 7.6. Vertical submersion, angled tip

APPENDIX A.8:

Water supply apparatus

SCHEMATIC:

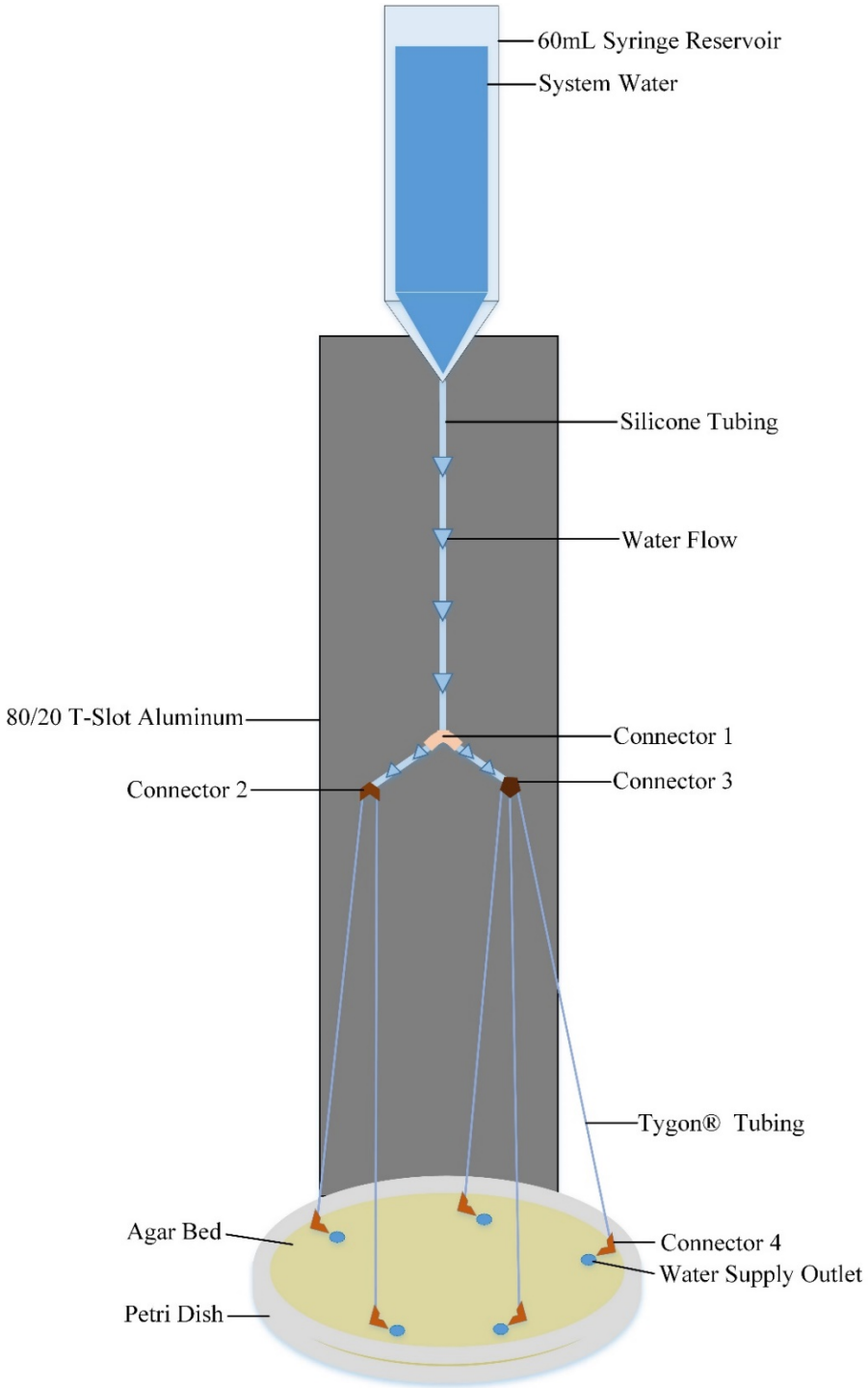


Figure 8.1.

MATERIALS:

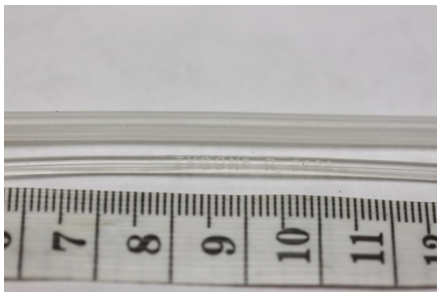


Figure 8.2. Silicone tubing (top) and Tygon® tubing (bottom)

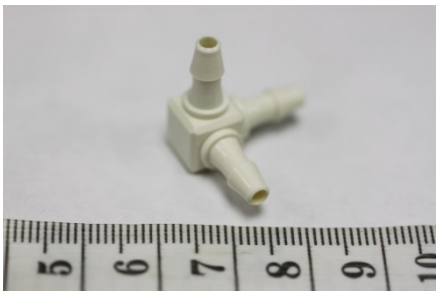


Figure 8.3. Connector 1

Figure 8.4. Connector 2

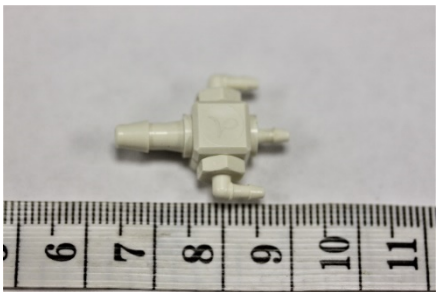


Figure 8.5. Connector 3

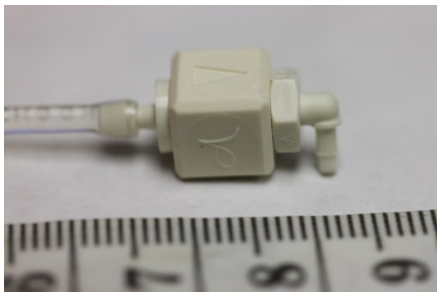


Figure 8.6. Connector 4

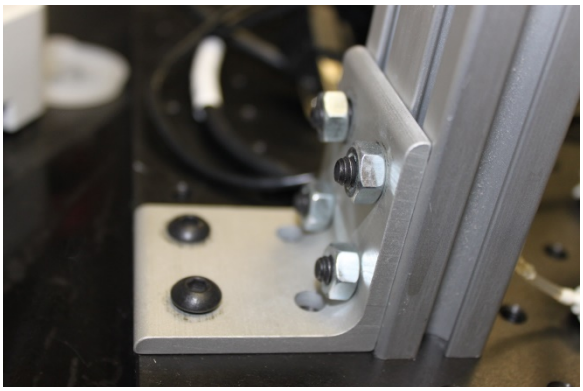


Figure 8.7. 80/20 T-slot aluminum attached to threaded stage with corner bracket, screws and nuts

APPENDIX A.9:

Reverse-mold agar well

SOLIDWORKS RENDERING (mm):

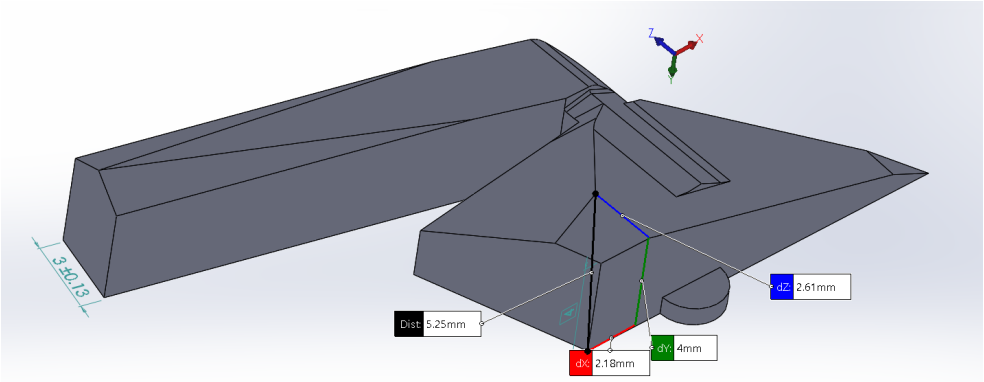


Figure 9.1. BOTTOM VIEW

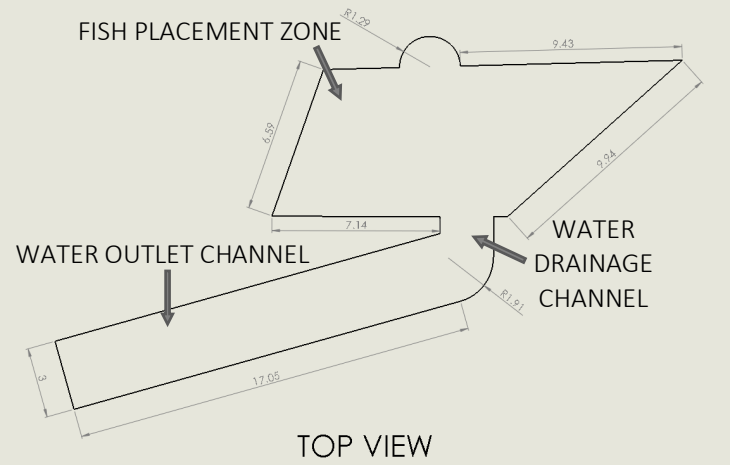


Figure 9.2.

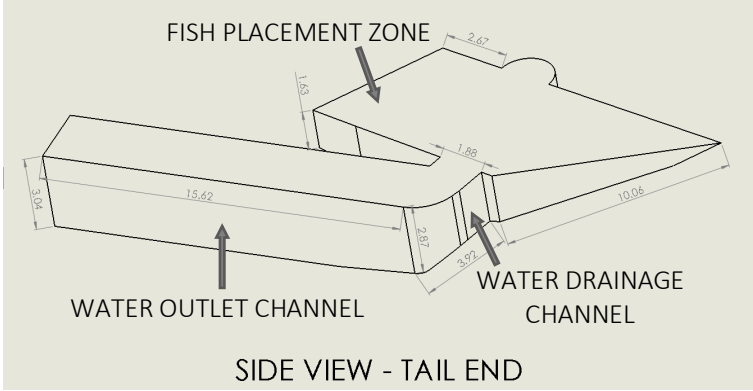


Figure 9.3.

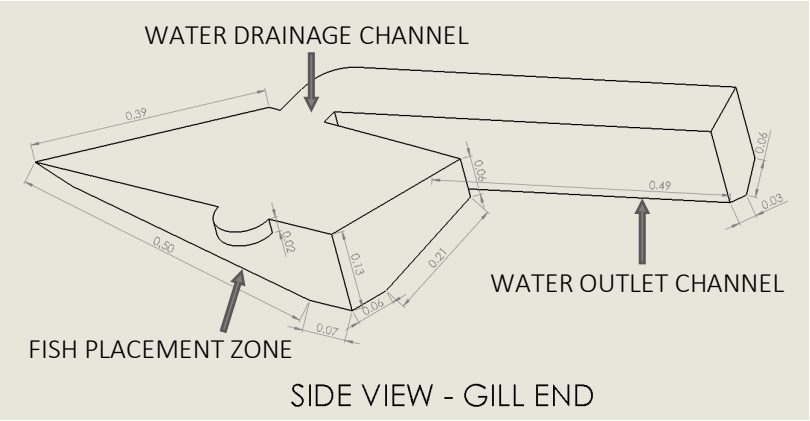


Figure 9.4.

3D-PRINTED PART (mm):

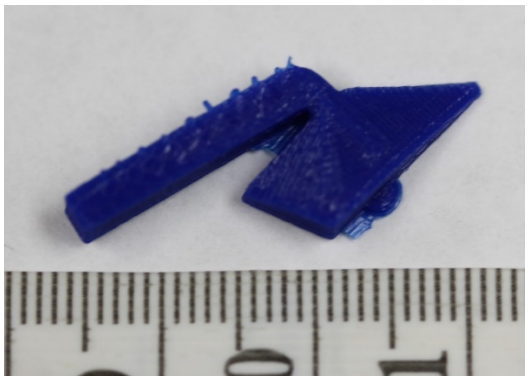


Figure 9.5. Bottom view – brim removed

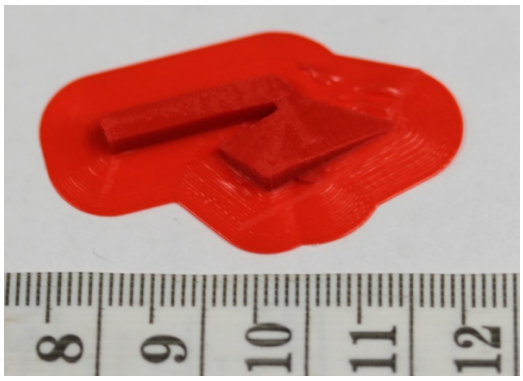


Figure 9.6. Bottom view – brim retained

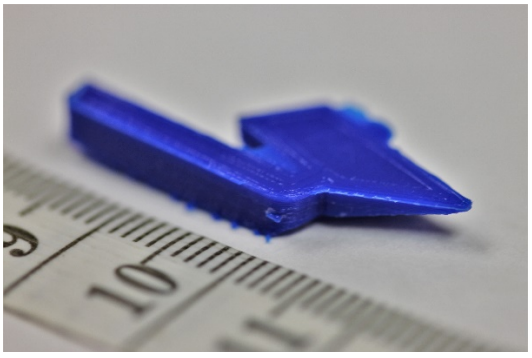


Figure 9.7. Side view – tail end

APPENDIX A.10:

Agar plate setup

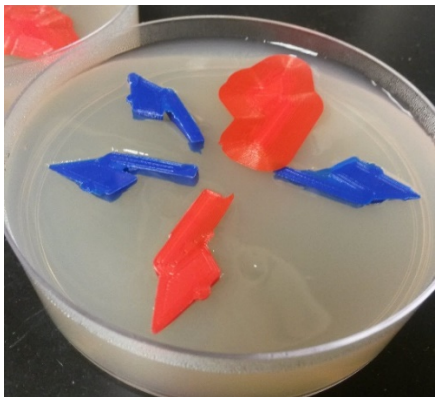


Figure 10.1. Reverse-mold applied to agar

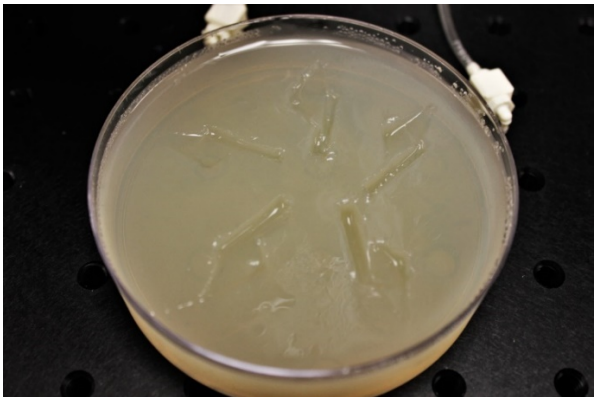


Figure 10.2. Reverse-mold removed

APPENDIX A.11:

Microincision practice on euthanized zebrafish

Red arrows indicate areas of interest, black arrows indicate locations on fish

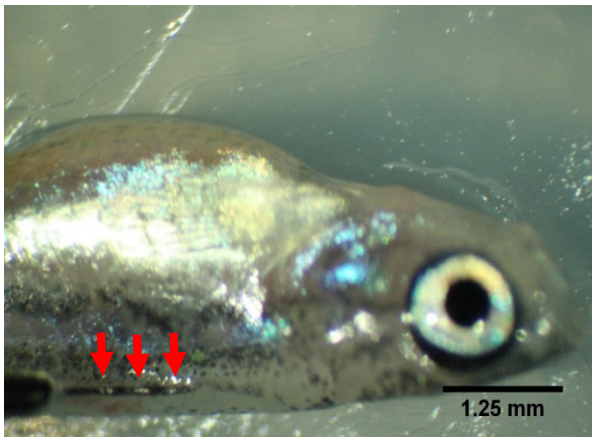


Figure 11.1. Red arrows indicate microincision performed with the vertical submersion, gradual-withdrawal tungsten needle (under epidermis)

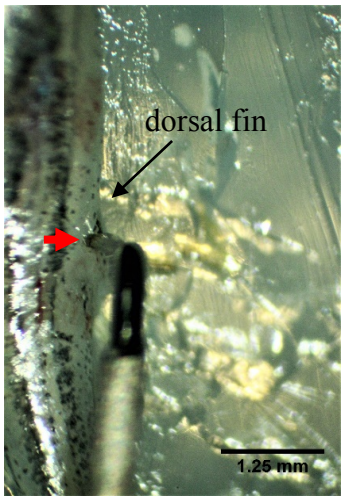


Figure 11.2. Red arrow: Tungsten hook inserted into dorsal line tissue

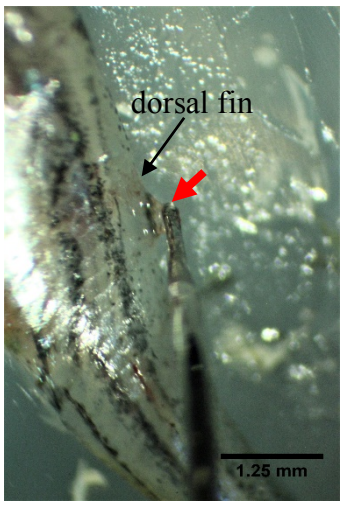


Figure 11.3. Red arrow: Angled tungsten needle lifting flap of epidermis

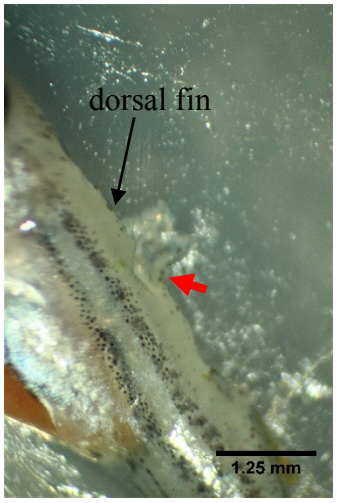


Figure 11.4. Red arrow: Pocket created by micromanipulator and 22.5° stab microscalpel

Implantation practice on euthanized zebrafish

Red arrows indicate areas of interest, black arrows indicate locations on fish

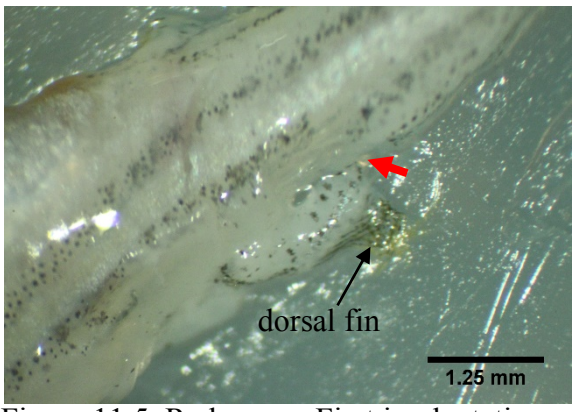


Figure 11.5. Red arrow: First implantation attempt using micromanipulator and manual insertion

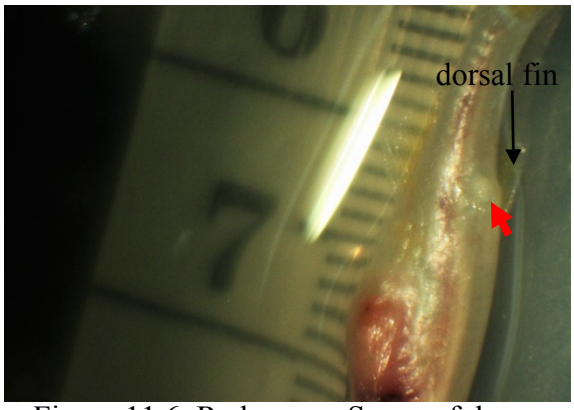


Figure 11.6. Red arrow: Successful insertion of square CNF implant ventral to the dorsal fin in 5 month- old Casper

APPENDIX A.12:

Operation setup

COMPONENTS:

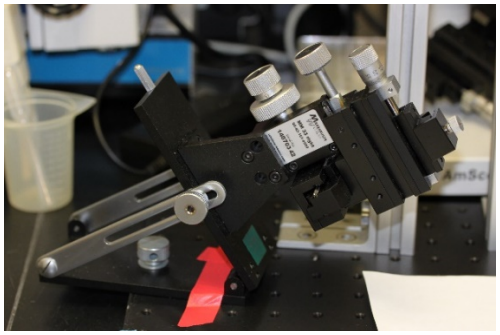


Figure 12.1. Micromanipulator

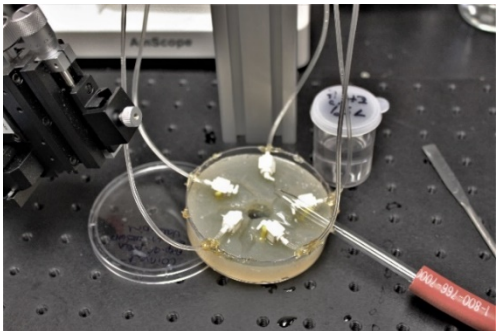


Figure 12.2. Agar plate/water supply apparatus

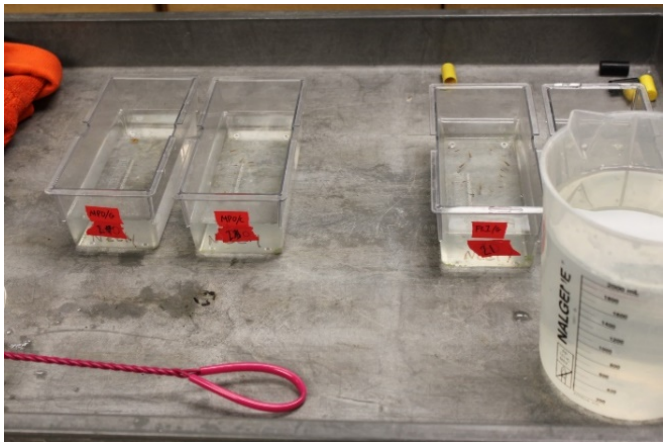


Figure 12.3. Zebrafish trial groups on standby for operation

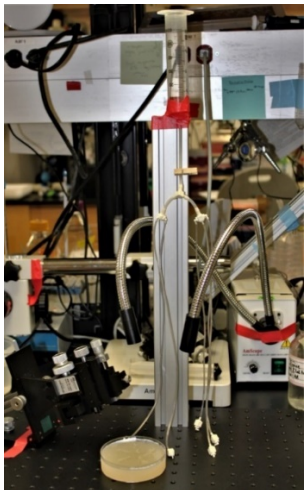


Figure 12.4. Total operation setup

Operation setup

INSTRUMENTS UTILIZED:

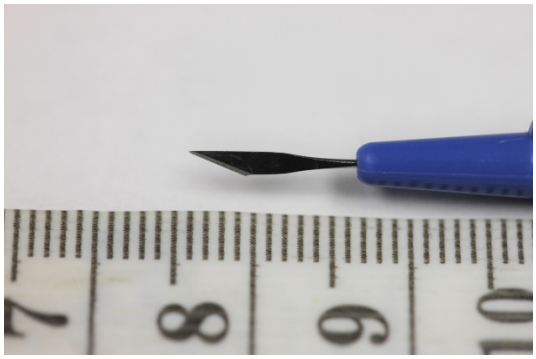


Figure 12.5. 22.5° Stab microscalpel

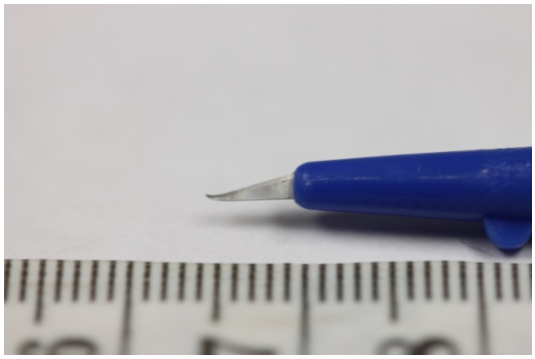


Figure 12.6. 15° Stab microscalpel, slightly bent

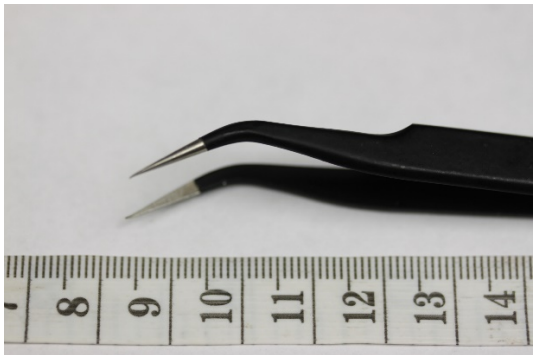


Figure 12.9. Vetus HRC40 #ESD-15 tweezers

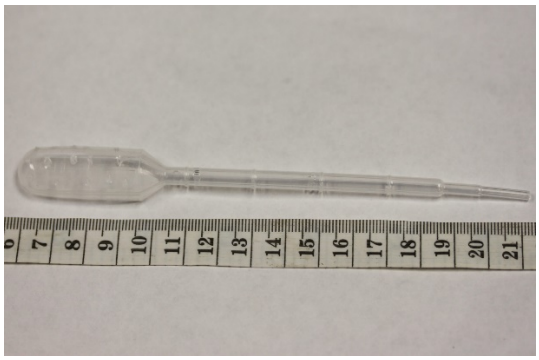


Figure 12.8. Punctured pipet scooping tool

APPENDIX A.13:

Imaging

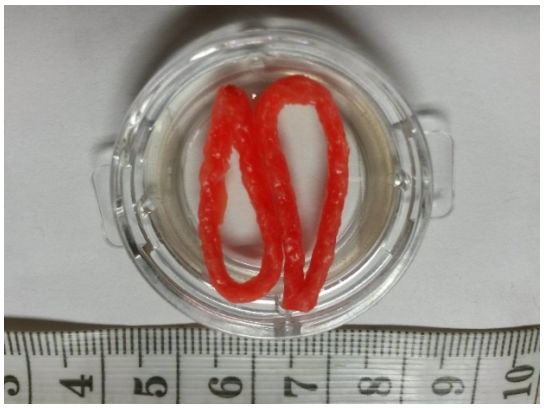


Figure 13.1. Delta T-dish (0.5mm) with waxed yarn barriers (cm)

Figure 13.2. Delta T-dish on stage adapter for fish (cm)

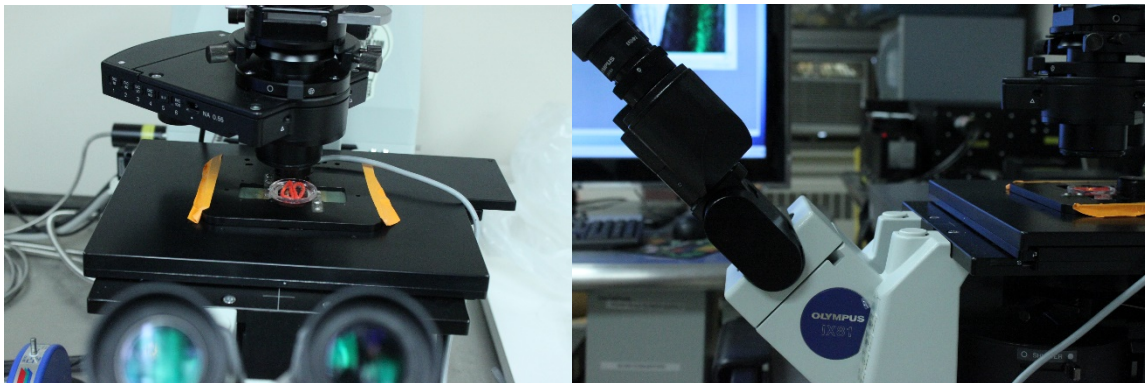


Figure 13.3. Imaging zebrafish using Delta T-dish/stage adapter setup with an Olympus IX81confocal microscope

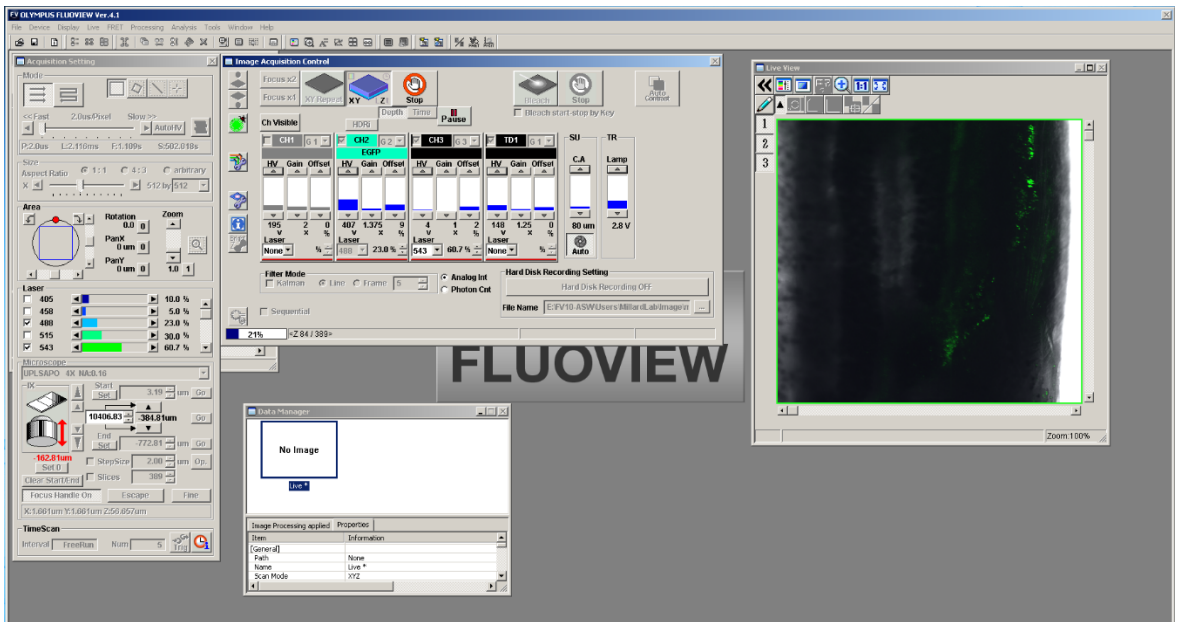


Figure 13.4. Olympus FLUOVIEW FV1000 program layout used for image acquisition

APPENDIX A.14:

Pigmentation of Casper crosses

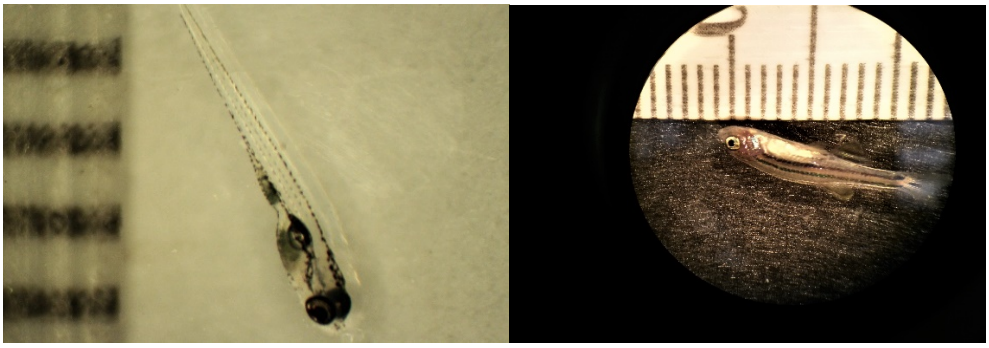


Figure 14.1. Casper / MPO: 3 weeks post-fertilization (left) and 10.5 weeks post-fertilization (right)

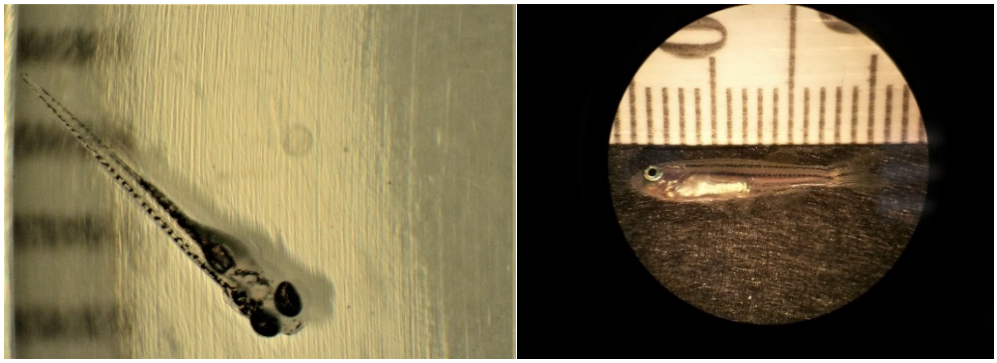


Figure 14.2. Casper / fli1: 3 weeks post-fertilization (left) and 10.5 weeks post-fertilization (right)

APPENDIX A.15:

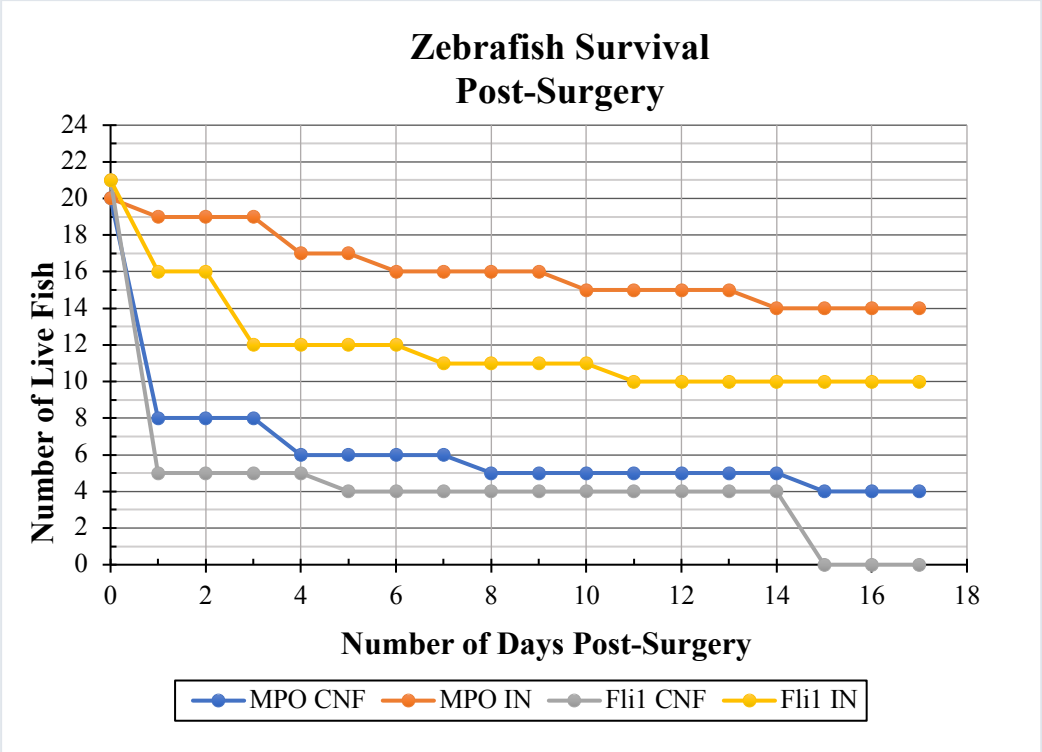


Figure 15. Post-surgery survival of Casper crosses by transgenic type and surgical trial. Key: 'MPO' denotes MPO-Casper transgenic fish, and 'Fli1' denotes fli1-Casper transgenic fish. 'IN' indicates incision-only trials, and 'CNF' indicates the CNF shard trials.

APPENDIX A.16

Post-surgical trends in fli1 Casper crosses

Control fli1

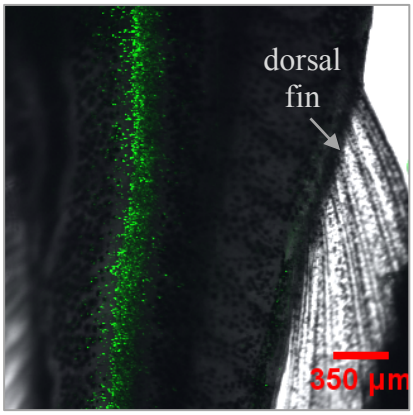


Figure 16.1. EGFP on vasculature (single slice) (composite)

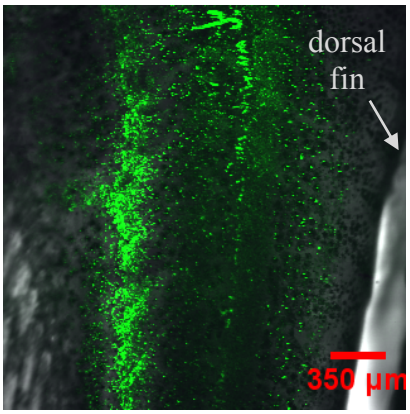


Figure 16.2. EGFP on vasculature

5 days post-surgery

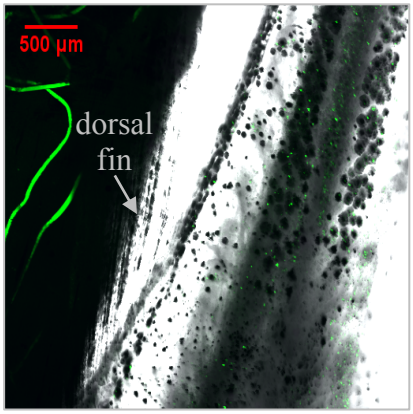


Figure 16.3. fli1-IN: Scattering of epithelial cells around vasculature, re-epithelialized incision

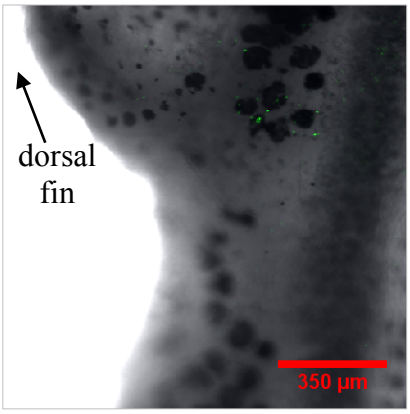
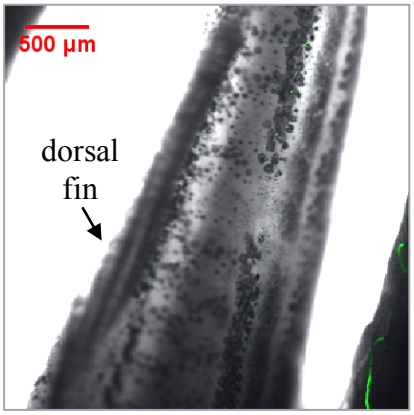
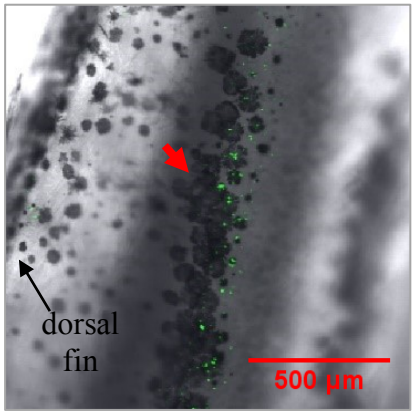


Figure 16.4. fli1-CNF: Empty pocket indicating lack of CNF retention

7 days post-surgery



16.5. fli1-IN: Very slight EGFP displayed on vasculature



16.6. Red arrow indicates some concentration of endothelial cells, no CNF retention

9 days post-surgery

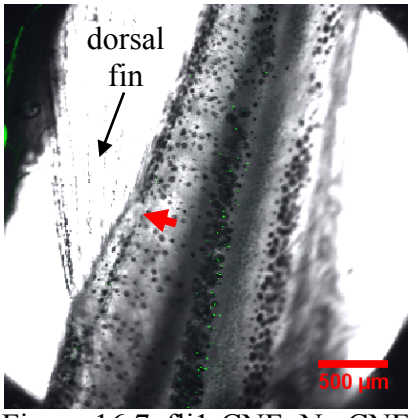


Figure 16.7. fli1-CNF: No CNF retention, re-epithelialized injury

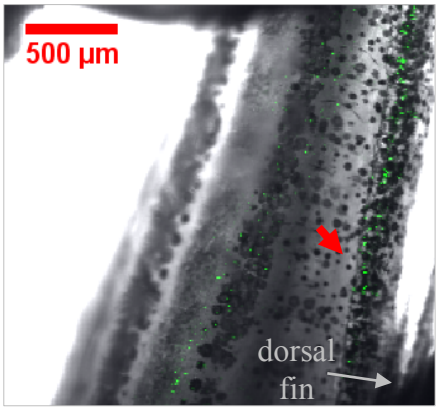


Figure 16.8. fli1-IN: Red arrow: re-epithelialized incision, endothelial cells concentrated along vasculature

15 days post-surgery

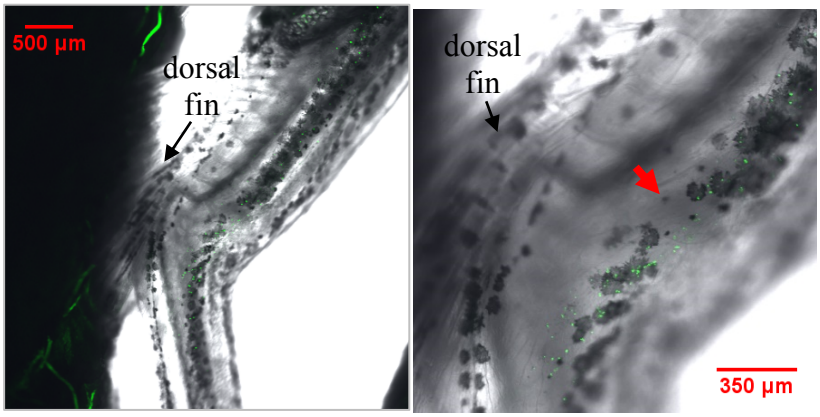


Figure 16.9. fli1-IN: Red arrow indicates endothelial cells viewed along vasculature, re-epithelialized injury with malformed tail; 15dps

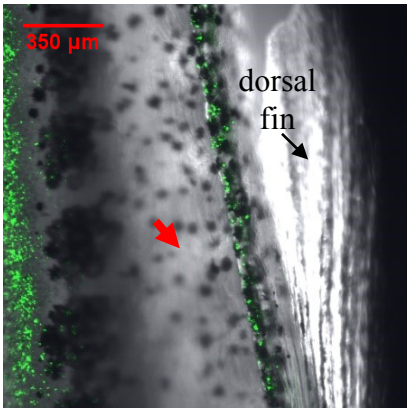


Figure 16.10. fli1-IN: Red arrow indicates high concentration of endothelial cells along vasculature, re-epithelialized incision

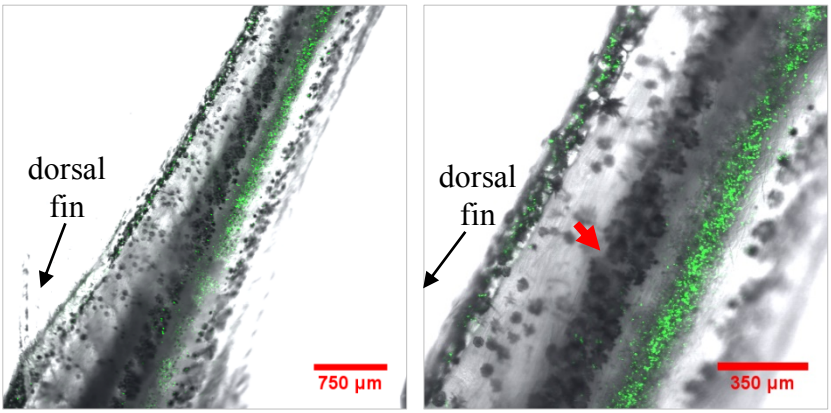


Figure 16.11. fli1-IN: High concentration of endothelial cells along vasculature but not around site of incision, indicated by red arrow

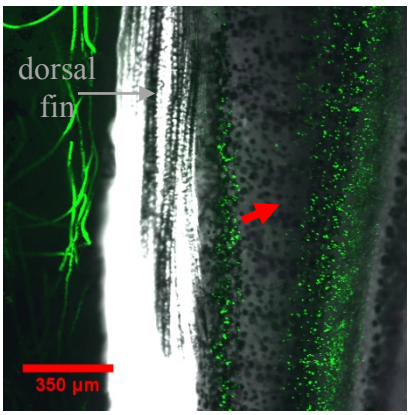


Figure 16.12. fli1-CNF: Red arrow: No implant retention, high concentration of endothelial cells confined to vasculature

APPENDIX A.17:

Post-surgical trends in MPO Casper crosses

Control MPO

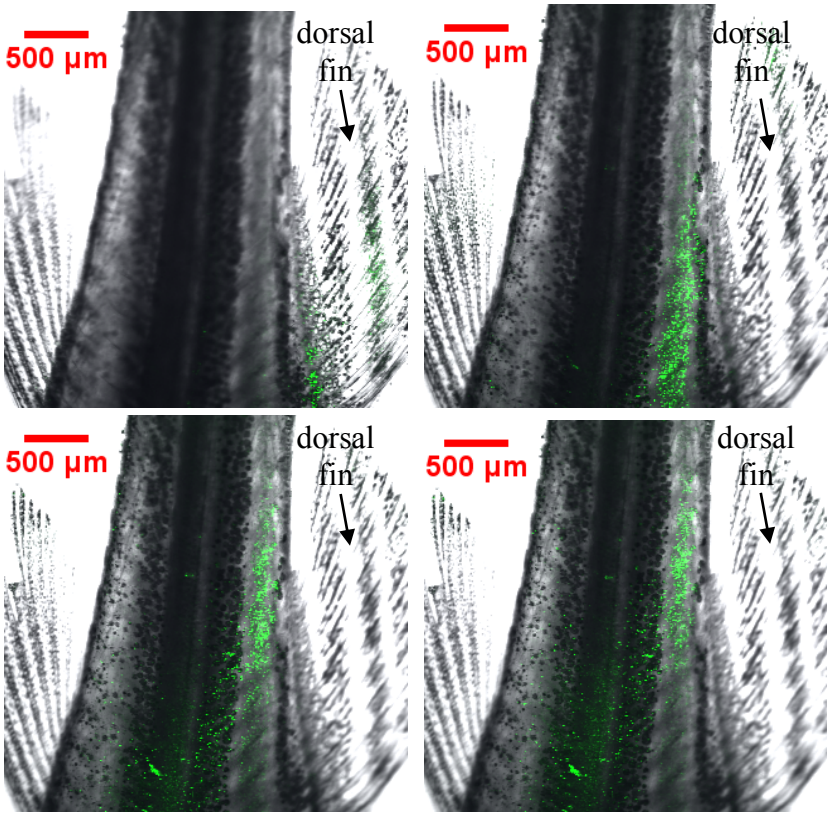


Figure 17.1. Concentration of neutrophils shown across multiple confocal image layers

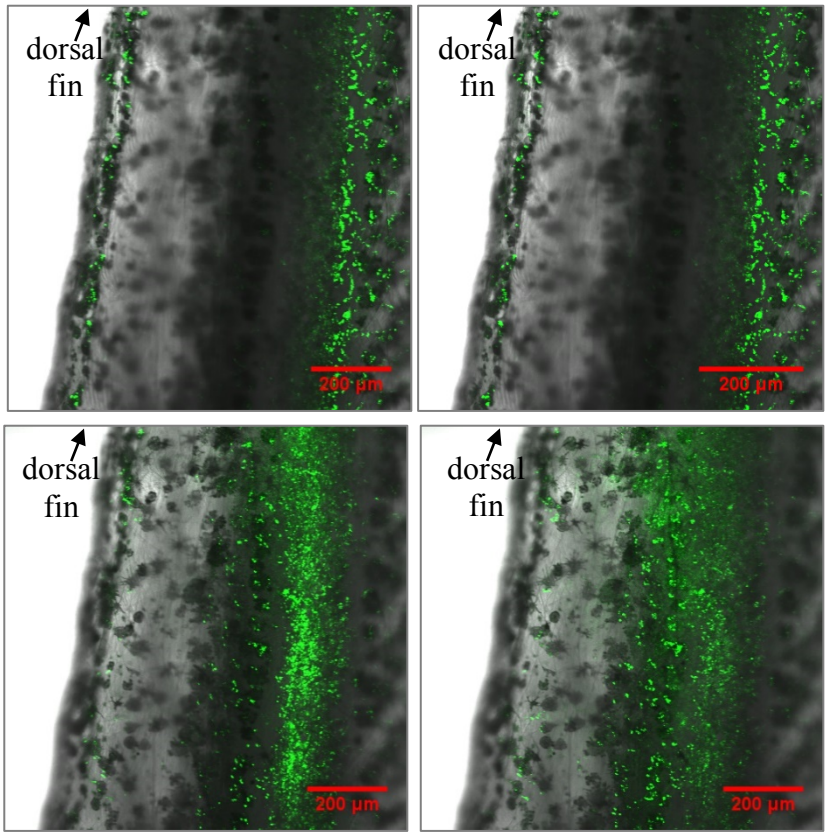


Figure 17.2. Control MPO: Concentration of neutrophils shown across multiple confocal image layers

4 days post-surgery

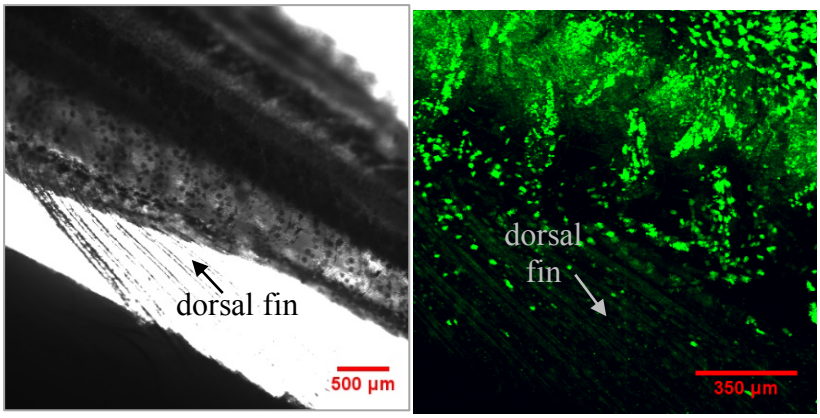


Figure 17.3. MPO-IN: Neutrophil concentration around site of incision

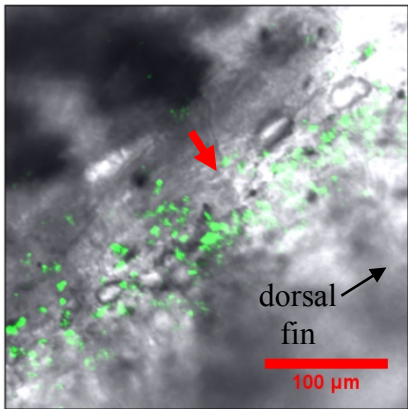


Figure 17.4. MPO-CNF: Red arrow indicates neutrophil concentration around implantation site; no CNF retention

6 days post-surgery

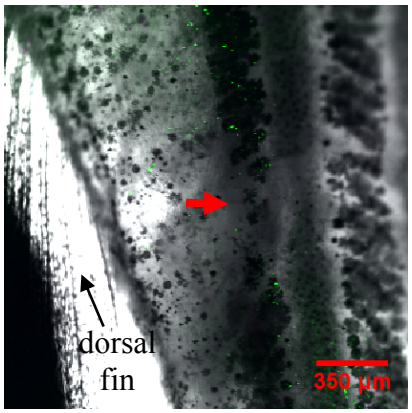


Figure 17.5. MPO-IN: Red arrow indicates pigment interruption, neutrophil concentration around the incision site

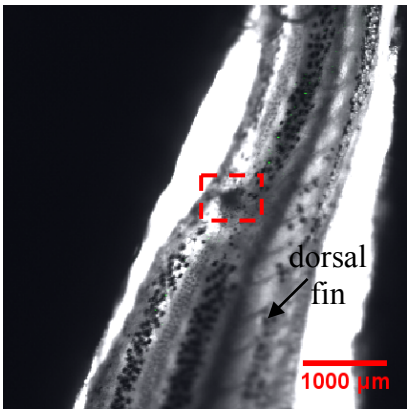


Figure 17.6. MPO- CNF: Box indicates retained CNF implant

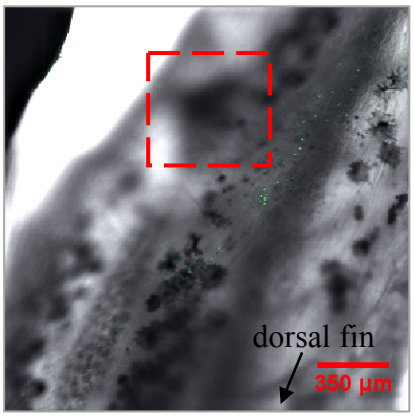


Figure 17.7. MPO-CNF: Red box indicates

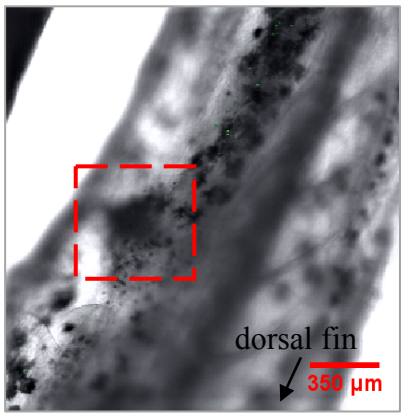


Figure 17.8. MPO-CNF: Red box

retained CNF, surrounding neutrophil concentration is present

indicates retained CNF, neutrophil concentration in same plane as CNF implant, determined by confocal imaging

8 days post-surgery

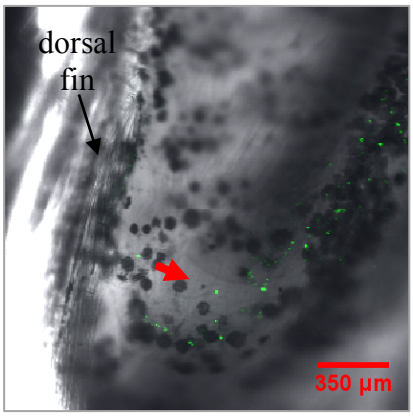


Figure 17.9. MPO-IN: Red arrow indicates neutrophil concentration around incision site

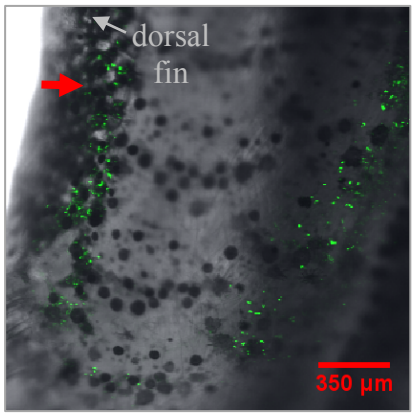


Figure 17.10. MPO-IN: Greater neutrophil concentration in layer superior to 17d.a

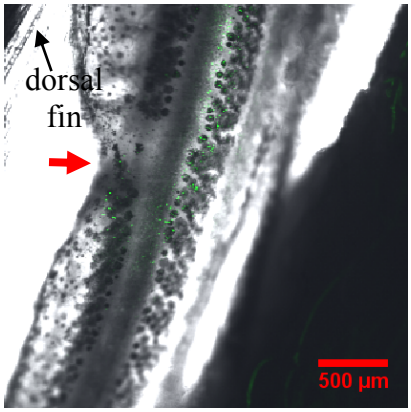


Figure 17.11. MPO-CNF: Some neutrophil concentration in pocket where CNF had fallen out

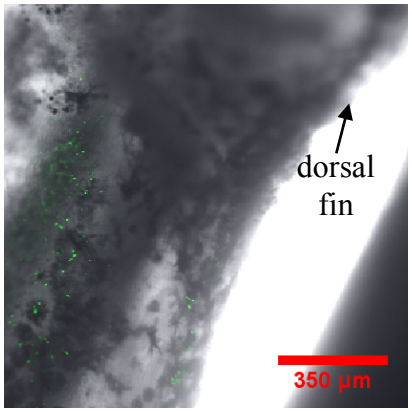


Figure 17.12. MPO-CNF: Close-up of empty CNF pocket, with surrounding neutrophil localization

14 days post-surgery

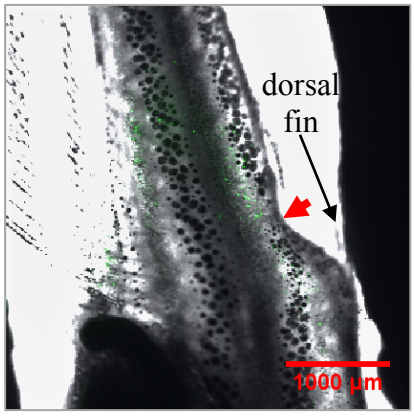


Figure 17.13. MPO-CNF: Red arrow points to pocket indicating lack of CNF retention, surrounding neutrophil concentration apparent

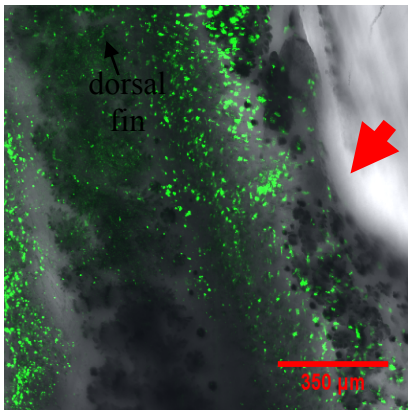


Figure 17.14. MPO-CNF: Close-up of 17.13; neutrophil concentration is more visible

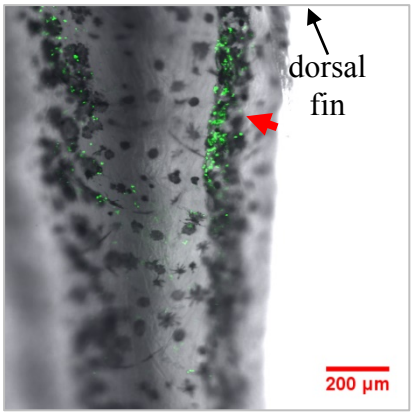


Figure 17.15. MPO-IN: Red arrow indicates re-epithelialized incision, some localized neutrophils remaining along incision site

APPENDIX B: PROTOCOLS

APPENDIX B.1:

PTU Preparation

Safety Measures: PTU:

Work under hood, wear PPE: gloves, coat, goggles

1. Add 50mg 1-phenyl 2-thiourea to a 50mL conical tube.
2. Add 20mL deionized water and vortex for 3 counts of 10 seconds.
3. Heat tube in water bath, vortexing as above every half hour until dissolved.
4. Filter through #1 Whatman in funnel or as a filter attachment with a 60mL syringe to dispense into larger container.
5. Bring final volume up to 50mL with deionized water.
6. Store at 4°C.

APPENDIX B.2:

Protocol for PTU administration on embryonic zebrafish

1. Place collected embryos in deep petri dish with 55mL egg water.
2. Add 0.63mL PTU solution.
3. Change solution at least once every 24 hours depending on number of embryos in each dish.

APPENDIX B.3:

Mesh basket assembly

Materials:

Nylon mesh

Sewing machine/thread

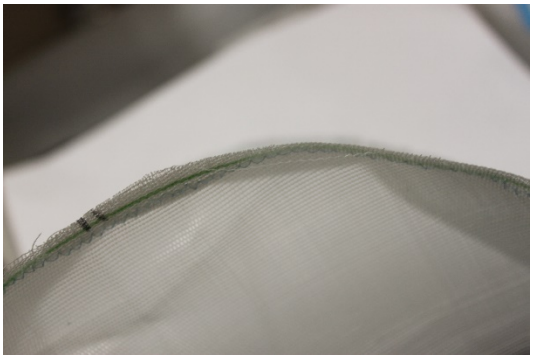
Pins

Scissors

Protocol:

1. Cut nylon mesh into three pieces: one 20” x 4” rectangle, two semicircles with radii of 7”.

2. Mark the centers, lengthwise, of all three pieces. Use the rounded edge of the semicircles and both 20” edges of the rectangle.
3. Match the center of one edge of the rectangle with the center of one of the semicircles; hold together with pins.
4. Working from the middle, join the curved edge of the semicircle piece with the edge of the rectangle and pin.
5. Sew with a zig-zag stitch with a 1/8”-1/4” allowance (a).
6. Repeat steps 3-5 with the other semicircle piece to finish basket (b).
7. Basket should fit into a 2.75L tank.



(a)

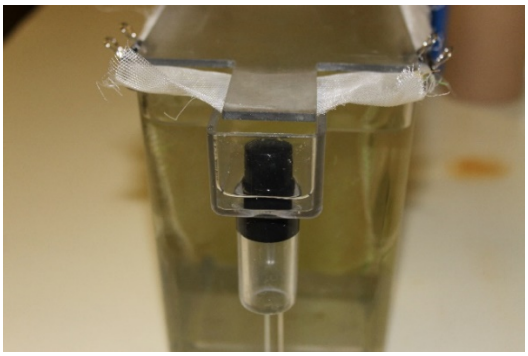
(b)

APPENDIX B.4:

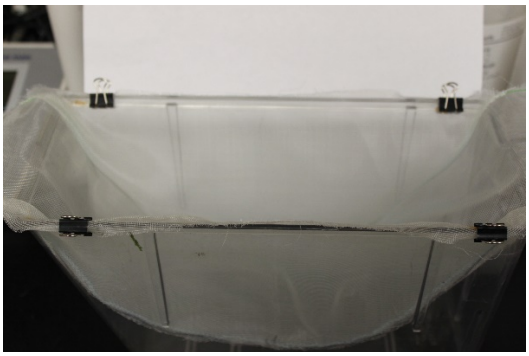
Protocol for PTU administration in juvenile zebrafish – basket method

1. Stopper the run-through outlet of a 2.75L tank to prevent leakage (a).
2. Wearing proper PPE, carefully measure 31.35mL of the PTU solution with a graduated cylinder and pipettor; add to tank.
3. Place tank under system water spout and fill tank three-quarters full with water.
4. Insert mesh basket and secure with mini binder clips, one by each tank corner (b).
5. Continue filling tank until water level reaches 1/2” below the rim of the tank.
6. Add fish and place plastic cover on top of tank (c, d).
7. If changing solution, fill a clean, empty tank with system water about three-quarters full.

8. Remove basket containing fish from the old solution and quickly place in tank from previous step.
9. Adjust water level if needed to cover the fish, gently rinsing the sides of the basket's interior to release any fish that may have gotten stuck to the mesh during transfer.
10. Bring the old tank to the floor drain and position parallel to the grates, gripping the outlet portion.
11. Stand behind the outlet and raise it to slowly pour the old PTU solution into the drain. This helps avoid splashes.
12. Quickly rinse the emptied tank with deionized water in the sink, then follow steps 2-5, replacing the mesh basket and fish from step 8.
13. Empty the extra tank, rinse with deionized water, and spray with perosan.
14. Dispose of tips in biohazard bag and wipe down all surfaces with perosan.

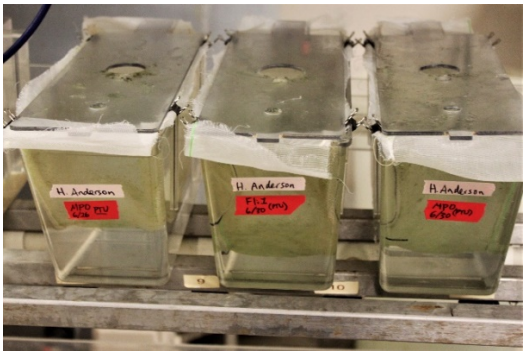


(a)



(b)

(c)



(d)

APPENDIX B.5:

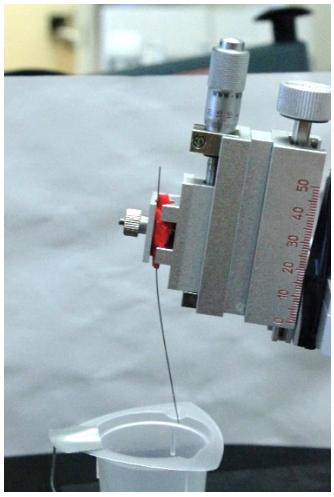
Electrolytic sharpening of tungsten wire (adapted from Moore and Kennedy ())

Materials:

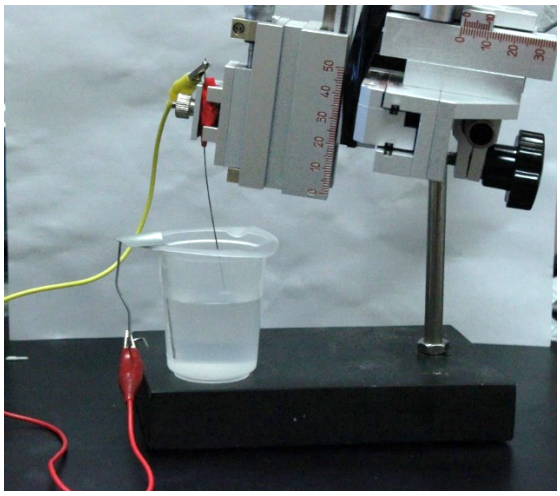
1M sodium hydroxide (NaOH) in deionized water
Deionized water
Tungsten wire (0.5-mm diameter)
Needle-nose pliers with wire cutter
Micromanipulator with ring-stand adapter
Ring stand or similar
Power supply (regulated direct current of at least 2 amps)
Electrode (paper clip)
Mini alligator clips

Protocol:

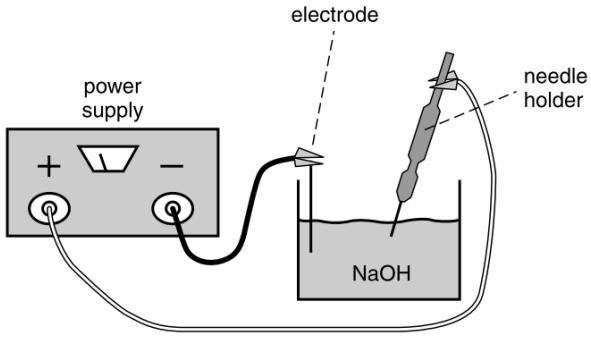
1. Position micromanipulator with ring-stand adapter on stand so that the wire will be facing down, providing support if needed.
2. Cut tungsten wire to desired length, at least 3-5cm, using wire cutters.
3. Bend the distal 1 cm of the wire to desired angle.
4. Secure tungsten wire in micromanipulator apparatus (a).
5. Make 1M NaOH solution with deionized water and put 75mL in a 125mL plastic container.
6. Assemble power supply, electrodes, 125mL container NaOH, mini alligator clips, and tungsten wire/micromanipulator apparatus as shown in (b) and (c).
7. Set power supply between 5 and 20V using direct current (d).
8. Immerse bent end of tungsten wire using the adjustments on the micromanipulator to lower it into the NaOH solution. Watch for bubbles on the electrode after a few seconds (e), then continue immersion until tip is <10 μm in diameter.
9. Rinse tungsten needles with deionized water before use.



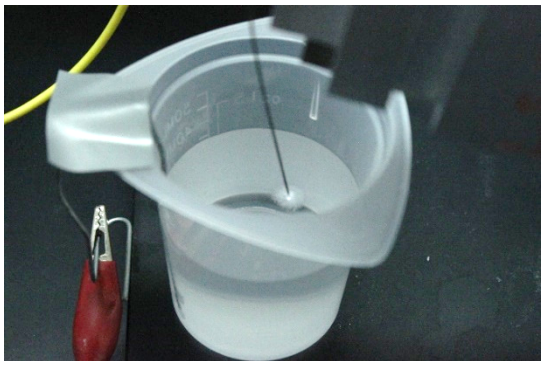
(a)



(b)



(c) Reproduced from Ref [30].



(d)

APPENDIX B.6:

Postsurgical Treatment Protocol

1. Add 2.3mL methylene blue to 97.7mL deionized water to make 100mL of a 2.3% solution.
2. Carefully break open one cephalexin capsule and weigh out 18.15mg powder into a small weigh boat.
3. Add cephalexin powder and 0.363mL methylene blue to empty holding tanks (stoppered).
4. Insert mesh baskets as in Appendix I, Steps 3-6.

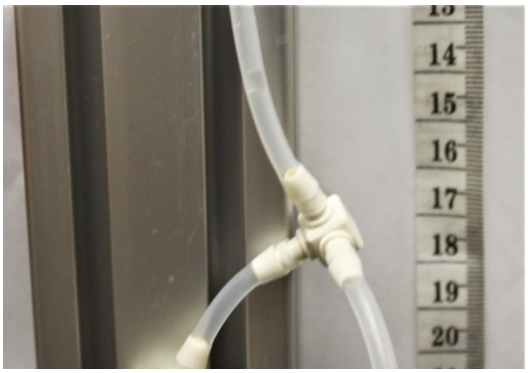
5. Administer treatment for 3 days total, changing solution daily by following the Appendix I, Steps 7-14 and the steps above. Abstain from feeding fish.
6. After final treatment, transfer zebrafish to regular run-through tanks and resume normal feeding.

APPENDIX C: TABLES

APPENDIX C.1:

Water supply apparatus

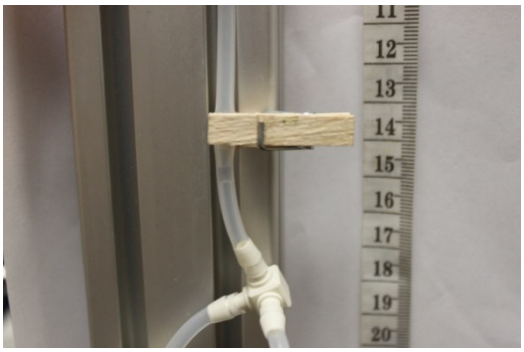
Flow rate of unclamped tube



TRIAL:	TIME (s):	WEIGHT (mg):	MASS RATE(mg/s):	FLOW RATE(mL/s):
1	60	32.043	0.534	0.534
2	60	34.980	0.583	0.583
3	60	33.150	0.553	0.553
4	60	32.847	0.547	0.547
5	60	32.979	0.550	0.550
Average Unclamped:			**0.553**	**0.553**

Table 1.1. Flow rate of unclamped tube

Flow rate of clamped tube



TRIAL:	TIME (s):	WEIGHT (mg):	MASS RATE (mg/s):	FLOW RATE (mL/s):
1	60	28.726	0.479	0.479
2	60	31.129	0.519	0.519
3	60	30.127	0.502	0.502
4	60	30.733	0.512	0.512
5	60	30.680	0.511	0.511
Average Clamped:			**0.505**	**0.505**

Table 1.2. Flow rate of clamped tube

APPENDIX C.2:

Water supply apparatus

MATERIAL SPECIFICATIONS:

ITEM:	DIMENSIONS:	QUANTITY:
80/20 T-slot Aluminum	46 x 5 x 2.5 cm	1
St. Steel 90° Corner Bracket	5 x 4.7 cm	1
socket head cap Screws (button head)	¼ -20 UNC x 5/8”	6
Hex nut	¼ -20 UNC	4

Syringe Reservoir	60 mL	1
Silicone Tubing	3/32"ID x 5/32"OD x 1/32" Wall	1 x 10 cm 2 x 4 cm
Tygon® s3-r306 Tubing	1/32"ID x 3/32"OD x 1/32" Wall	5 x 35 cm
Connector 1	*(b)*	1
Connector 2	*(c)*	1
Connector 3	*(d)*	1
connector 4	*(e)*	5
Threaded Stage		1

Table 2. Material specifications

APPENDIX D.1:

IACUC approval notice

From: Paula Portalatin <paula.portalatin@maine.edu>
Date: June 21, 2017 at 3:13:16 PM EDT
To: Paul Millard <paul.millard@maine.edu>
Subject: Protocol A2017-05-01 - Approval

Protocol #: A2017-05-01
Protocol Title: Performing microincisions for the implantation of nanocellulose in the juvenile zebrafish model
PI: Paul Millard
Species/# Approved: Zebrafish/270
Approval Period: 6/21/2017-6/20/2020

Dear Paul,

The above referenced protocol has been approved by the University of Maine IACUC. As a courtesy the IACUC Office will generally send out reminders for annual and de novo reviews however, it is ultimately the responsibility of the PI to ensure that the protocol is renewed on time.

All of the proposed methods, procedures, and conditions have been approved AS STATED IN THE PROTOCOL APPLICATION. The IACUC must approve any changes or deviations from the approved protocol prior to being initiated.

University of Maine Animal Welfare Assurance #: A3754-01
The University of Maine is registered as a research facility in accordance with the U.S. Department of Agriculture Animal Welfare Act and the Public Health Service Policy on the Humane Care and Use of Laboratory Animals. The University of Maine holds the Office of Laboratory Animal Welfare (OLAW) of the National Institutes of Health assurance for vertebrate animals used in research, teaching and outreach.

The Animal Welfare Assurance (1) confirms the commitment that the University of Maine will comply with the PHS Policy, with the Guide for the Care and Use of Laboratory Animals, and with the Animal Welfare Regulation; (2) describes the institution's program for animal care and use; and (3) designates the institutional official responsible for compliance.

I have attached the final approved version to this email. A cage card is also attached; please post near or on the cages. Thank you.

Sincerely,
Paula Portalatin, M. Ed., CPIA
Research Compliance Officer II
Office of Research & Sponsored Programs
University of Maine
Room 402 Corbett Hall
Orono, Maine 04469-5717
(207) 581-2657

APPENDIX D.2:

Completed IACUC protocol

Version October 2017

PROTOCOL NUMBER: A2017-05-01

PI/INSTRUCTOR NAME: Paul Millard

PROTOCOL TITLE: Performing microincisions for the implantation of nanocellulose in the juvenile zebrafish model.

UNIVERSITY OF MAINE

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

PROTOCOL REVIEW FORM FOR

RESEARCH, TEACHING OR PILOT STUDIES

This protocol form is for research, teaching, or pilot studies using vertebrate animals. Husbandry (breeding and production) of vertebrates solely for the purpose of supplying animals for research, teaching or pilot studies requires a different documentation form (found on the IACUC website).

GENERAL INSTRUCTIONS: The Institutional Animal Care and Use Committee (IACUC) consists of scientists from several disciplines as well as non-scientists, members of the University community, and persons who have no other affiliation with the University than as members of the Committee. The protocol should therefore be described in terms understandable by an audience of educated nonspecialists.

Please submit the completed protocol to the Institutional Animal Care and Use Committee via email to umric@maine.edu. The form is due TWO weeks prior to a scheduled IACUC meeting. NOTE: The Principal Investigator (PI) MUST submit the protocol. Another faculty member (no students) may submit the protocol on behalf of the PI with documentation of an email exchange that the PI has read and approves. We require this because the PI is ultimately responsible for the content of the protocol submission.

The meeting dates are posted at: http://www.umaine.edu/research/research-compliance/institutional-animal-care-and-use-committee-iacuc/meeting-schedule-and-protocol-due-dates/. Protocols received late will be held until the next month's meeting. Please call Gayle Jones (1-1498) or Paula Portalatin (1-2657) if you have questions.

The Principal Investigator or Instructor must justify the ethical costs of using live vertebrate animals by demonstrating a reasonable expectation that such usage will contribute to the advancement of knowledge which may eventually benefit humankind and/or animals. The Principal Investigator or Instructor must further demonstrate that he or she has applied the concepts of "alternatives" in designing the protocol. The term "alternatives" includes three components: replacement (using nonvertebrate animals, cell cultures, tissues from slaughter or autopsy, or nonanimal systems); reduction (in the number of animals used); and refinement (of design and methods to reduce pain and stress to animals used as well as ensuring that the number of animals used is optimal for the analysis proposed).

1. This form is for New Protocols. If you wish to amend a previously approved protocol, **see instructions/form for submitting an amendment.**

2. Principal Investigator/Instructor and Co-Investigator(s) (NOTE: The Principal Investigator or Instructor must be a faculty member or professional staff):

PI Name: Dr. Paul Millard

Campus Address: Jenness Hall

Phone: 207-735-6037

Email: hanna.anderson@maine.edu

Co-PI Name: Dr. Con Sullivan

Campus Address: 284 Hitchner Hall

Phone: 207-581-2809

Email: con.sullivan@maine.edu

Will any non-UMaine personnel handle or have responsibilities for the animals (i.e., collaborations)? ☒ No ☐ Yes. If yes, please name personnel below with his/her affiliation. An Inter-Institutional Agreement may be required. See: https://umaine.edu/research/resource/inter-institutional-agreement/

3. Title of project:

Performing microincisions for the implantation of nanocellulose in the juvenile zebrafish model.

4. Date protocol or amendment will go into effect:

(REMINDER: Activities may not begin until IACUC approval and amendments are only for period of original protocol.)

May 2017

5. Funding agency for project, if applicable:

Please attach the vertebrate animal (VA) section/methods section from the proposal. If multiple agencies are involved, please send only the VA sections that specifically relate to this protocol.

N/A

6. Briefly describe the (check appropriate category) ☒ research, ☐ teaching, or ☐ pilot study objectives **(not procedures)** that involve use of animals. Describe these objectives in non-technical language. Do not paste in sections of grant proposals.

The main objective of this research project is to utilize the zebrafish animal model system to assess biomaterials with potential for use in medical implants. The biomaterial in question is dense nanocellulose, specifically cellular nanofibrils (CNFs), in solid form created in the Chemical and Biological Engineering Department facilities at the University of Maine. One aspect of the project will focus on methods for implanting the novel nanocellulose material into zebrafish to evaluate the potential of this material for use in prosthetics in humans, as zebrafish contain a similar immune response that can be readily observed.(17) This project holds considerable potential for the advancement of naturally-sourced implantable biomaterials due to the biocompatibility, durability, and availability of nanocellulose. With a porous composition, nanocellulose enables ample cellular integration, permitting a more secure implant. Variations in structure, including the density and specific shape of the nanocellulose shard, affect the integration into tissues, therefore optimization of these parameters is crucial for the improvement of existing implant formats. Implantation will likely require micro-incisions to be made on the model, and/or physical modification of the nanocellulose to permit insertion. A new approach for creating micro-incisions in the zebrafish may need to be developed, as zebrafish are delicate and are only a few millimeters in length, rendering conventional techniques difficult to implement while maintaining the survival of the animal.

When implantation has been accomplished, immune responses and other changes in the condition of surrounding tissue will be monitored and recorded by imaging, including bright-field and fluorescence microscopy. MPO zebrafish (GFP-tagged neutrophils) will be utilized for the visualization and quantification of neutrophils migrating to the site of implantation. The amount of neutrophils that migrate will indicate whether the nanocellulose has been rejected or accepted by the model, providing insight into whether it remains inert when introduced to tissue. The observation of cellular integration of the nanocellulose in the model will also be conducted through the use of fli1 zebrafish (GFP-labeled vasculature), as well as the microscopy methods listed above.

7. Describe how this use of animals contributes to the advancement of knowledge that may eventually benefit humankind and/or animals

During recent years, zebrafish have become widely implemented in biomedical research concerning immunological responses. This is primarily due to the many advantages that *Danio rerio* offer, such as rapid development and the presence of both

innate and adaptive immune responses, where activation of the innate response occurs within 30hpf,(17) and the adaptive response develops between 4-6 weeks post-fertilization.(20) This time period between the two types of responses allows extended observation of the intricacies of the innate response of the model. In this experiment, the implantation will be performed after 96hpf for an initial observation of innate response by imaging the neutrophil migration to the implantation site with fluorescence microscopy on MPO zebrafish, which express GFP on their neutrophils. Fluorescence microscopy will also be conducted on fli1 zebrafish, which express GFP throughout their vasculature, to assess integration of the implant into the tissue.(25,26)

Neutrophils travel to areas of inflammation in order to phagocytose pathogens or microorganisms, and additional effects that their behavior may impart on the overall immune response are still being studied.(23) In this case, the migration of neutrophils towards the implanted nanocellulose will be modeled, including their interaction with the implant material, to observe its biocompatibility. As the immune responses of zebrafish have been compared to those of mammals, any observed behavior in this study may be applied to the potential immune response of other animals/humans towards nanocellulose in its solid form.(17) As described in Question 6, nanocellulose is a readily-available, naturally-sourced material that is paving the way as a sustainable biomaterial alternative that can be optimized for specific biomedical applications, particularly as a replacement for conventional polymer materials.(7)

1. Arizona Game and Fish Department. Fish Tagging and Marking Techniques [Internet]. 2013 [cited 2017 May 9]. Available from: http://www.azgfd.gov/w_c/Fish_Tagging_Marking_Techniques.shtml/

2. Sciences B. PIT Tagging : Simple Technology at Its Best. 2017;54(5):447–54.

3. Baras E, Malbrouck C, Houbart M, Kestemont P, Melard C. The effect of PIT tags on growth and physiology of age-0 cultured Eurasian perch Perca flu Õ iatilis of variable size. 2000;

4. Rompen E. The effect of material characteristics , of surface topography and of implant components and connections on soft tissue integration : a literature review. 2006;17:55–67.

5. Bo O, Pihlajama H. Clinical biocompatibility of biodegradable orthopaedic implants for internal " xation : a review. 2000;21.

6. Ambrose CG, Clanton TO. Bioabsorbable Implants : Review of Clinical Experience in Orthopedic Surgery. Ann Biomed Eng. 2006;32(1):171–7.

7. Jorfi M, Foster EJ. Recent advances in nanocellulose for biomedical applications. J Appl Polym Sci. 2015;132(14).

8. Morán JI, Alvarez VA, Cyras VP, Vázquez A. Extraction of cellulose and

preparation of nanocellulose from sisal fibers. Cellulose. 2008;15(1):149–59.

9. Lin N, Dufresne A. Nanocellulose in biomedicine: Current status and future prospect. Eur Polym J [Internet]. 2014;59:302–25. Available from: http://www.sciencedirect.com/science/article/pii/S0014305714002493

10. Torres FG, Troncoso OP, Torres C, Grande CJ. Advances in Natural Polymers. Adv Struct Mater [Internet]. 2013;18:21–54. Available from: http://www.scopus.com/inward/record.url?eid=2-s2.0-84874283870&partnerID=tZOtx3y1

11. Abraham E, Deepa B, Pothan LA, Jacob M, Thomas S, Cvelbar U, et al. Extraction of nanocellulose fibrils from lignocellulosic fibres: A novel approach. Carbohydr Polym [Internet]. 2011;86(4):1468–75. Available from: http://dx.doi.org/10.1016/j.carbpol.2011.06.034

12. Prakash P, Kasaby HE, Aggarwal RK, Humfrey S. Microincision bimanual phacoemulsification and Thinoptx implantation through a 1.70 mm incision. Eye (Lond). 2007;21(2):177–82.

13. Fine IH, Hoffman RS, Packer M. Optimizing refractive lens exchange with bimanual microincision phacoemulsification. J Cataract Refract Surg. 2004;30(3):550–4.

14. Ahoor M, Sorkhabi R. Intraocular Foreign Body Removal by 23-Gauge Micro Incision Vitrectomy Surgery and Back Flush Flute Needle: A Case Series Study. Glob J Health Sci [Internet]. 2016;9(5):262. Available from: http://www.ccsenet.org/journal/index.php/gjhs/article/view/56079

15. Sambasivarao S V. OCT-Documented incision features and natural history of clear corneal incisions used for bimanual microincision cataract surgery. Cornea. 2013;18(9):1199–216.

16. Gerlach GF, Morales EE, Wingert RA. Microbead Implantation in the Zebrafish Embryo. J Vis Exp [Internet]. 2015;(101):e52943. Available from: http://www.jove.com/video/52943/microbead-implantation-in-the-zebrafish-embryo

17. Brittijn SA, Duivesteijn SJ, Belmamoune M, Bertens LFM, Bitter W, De Bruijn JD, et al. Zebrafish development and regeneration: New tools for biomedical research. Int J Dev Biol. 2009;53(5–6):835–50.

18. Richardson R, Slanchev K, Kraus C, Knyphausen P, Eming S, Hammerschmidt M. Adult zebrafish as a model system for cutaneous wound-healing research. J Invest Dermatol [Internet]. 2013;133(6):1655–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23325040%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3644348

19. Chang W, Hwang P. Development of Zebrafish Epidermis. 2011;214(Part C):205–14.

20. Sullivan C, Kim CH. Zebrafish as a model for infectious disease and immune function. Fish Shellfish Immunol. 2008;25(4):341–50.

21. Lambris JD. Current Topics in Innate Immunity II.

22. Mathias JR, Perrin BJ, Liu T, Kanki J, Look AT, Huttenlocher A. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish flammation is a critical process during normal im-. 2006;80(December).

23. Rosales C, Demaurex N, Lowell CA, Uribe-querol E. Neutrophils : Their Role in Innate and Adaptive Immunity. 2016;2016:2–4.

24. Developmental stage data for this paper were retrieved from the Zebrafish Information Network (ZFIN); University of Oregon; Eugene; OR 97403-5274; URL: http://zfin.org/; 9/20/17.

25. Renshaw S, Loynes C. A transgenic zebrafish model of neutrophilic inflammation. Blood… [Internet]. 2006;108(13):3976–8. Available from: http://bloodjournal.hematologylibrary.org/content/108/13/3976.short

26. Lawson ND, Weinstein BM. In Vivo Imaging of Embryonic Vascular Development Using Transgenic Zebrafish. Dev Biol [Internet]. 2002;248(2):307–18. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0012160602907116

27. White RM, Sessa A, Burke C, Bowman T, Leblanc J, Ceol C, et al. Resource Transparent Adult Zebrafish as a Tool for In Vivo Transplantation Analysis. 2008;(February):183–9.

28. Karlsson J, von Hofsten J, Olsson P-E. Generating Transparent Zebrafish: A Refined Method to Improve Detection of Gene Expression During Embryonic Development. Mar Biotechnol [Internet]. 2001;3(6):0522–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14961324%5Cnhttp://link.springer.com/10.1007/s1012601-0053-4

29. Tobias CW, Eisenberg M. IN ELECTROLYSIS-- REVIEW. 99(12):359–65.

30. Moore SW, Kennedy TE. Dissection and culture of embryonic spinal commissural neurons. Curr Protoc Neurosci. 2008;(SUPPL. 43).

31. Conrad GW, Bee JA, Roche SM, Teillet MA. Fabrication of microscalpels by electrolysis of tungsten wire in a meniscus. J Neurosci Methods. 1993;50(1):123–7.

32. Fang P, Lin JF, Pan HC, Shen YQ, Schachner M. A Surgery Protocol for Adult

Zebrafish Spinal Cord Injury. J Genet Genomics [Internet]. 2012;39(9):481–7. Available from: http://dx.doi.org/10.1016/j.jgg.2012.07.010

33. OLYMPUS. Research Inverted System Microscope Ix71/Ix81 Ix2 Series. In: Manual.

34. Wikki Stix One of a Kind Creatables [Internet]. Available from: https://www.wikkistix.com/

35. Hachicho N, Reithel S, Miltner A, Heipieper HJ, Küster E, Luckenbach T. Body mass parameters, lipid profiles and protein contents of zebrafish embryos and effects of 2,4-dinitrophenol exposure. PLoS One. 2015;10(8):1–19.

36. Avella MA, Place A, Du SJ, Williams E, Silvi S, Zohar Y, et al. Lactobacillus rhamnosus Accelerates Zebrafish Backbone Calcification and Gonadal Differentiation through Effects on the GnRH and IGF Systems. PLoS One. 2012;7(9).

37. Hohn C, Petrie-Hanson L. Evaluation of visible implant elastomer tags in zebrafish (Danio rerio). Biol Open [Internet]. 2013;2(12):1397–401. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3863425&tool=pmcentrez&rendertype=abstract

38. Peng S, Yao L, Cui C, Zhao H, Liu C, Li Y, et al. Semaphorin4D promotes axon regrowth and swimming ability during recovery following zebrafish spinal cord injury. Neuroscience [Internet]. 2017;351:36–46. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0306452217301963

39. Collymore C, Tolwani A, Lieggi C, Rasmussen S. Efficacy and safety of 5 anesthetics in adult zebrafish (Danio rerio). J Am Assoc Lab Anim Sci. 2014;53(2):198–203.

40. Huang W-C, Hsieh Y-S, Chen I-H, Wang C-H, Chang H-W, Yang C-C, et al. Combined Use of MS-222 (Tricaine) and Isoflurane Extends Anesthesia Time and Minimizes Cardiac Rhythm Side Effects in Adult Zebrafish. Zebrafish [Internet]. 2010;7(3):297–304. Available from: http://www.liebertonline.com/doi/abs/10.1089/zeb.2010.0653

8. Total Animals and Pain Classification: Tabulate the total number of animals per species, life stage (e.g., larval, adult, all) and USDA pain classification. Indicate if these individuals are genetically modified (e.g., knock-out or transgenic). Individuals should be accounted for only once, under the highest pain classification planned for their use (see Appendix 1 for classification definitions and examples). Breeding and maintenance colonies used to produce or hold study subjects are generally not included in these numbers, unless this protocol requires significant deviations from approved husbandry practices (see husbandry protocol for

associated colony). Any future increases to these numbers require an approved amendment. (NOTE: to add rows, right-click within table, click on "insert" and choose "insert rows above" or "insert rows below")

Species **(Scientific Name/Common Name)**	**Stage(s)**	**USDA Class** **(B, C, D or E)**	**GMO (Y/N)**	**3 Year Total**
Danio rerio/zebrafish: MPO (GFP-tagged neutrophils)	Juvenile (1.5-2 months pf)	C	Y	30
Danio rerio/zebrafish: MPO (GFP-tagged neutrophils)	Juvenile (1.5-2 months pf)	D	Y	90
Danio rerio/zebrafish: TG (fli1:EGFP)	Juvenile (1.5-2 months pf)	D	Y	150
PROJECT TOTAL:				270

Mandatory Requirements for Classification D or E:

a) Veterinary Consultation: A consultation is required before the protocol will be formally considered for review. Please email or phone Dr. James Weber (1-2774, jaweber@maine.edu) with a description of the proposed procedures.

Date of veterinary consult: 4/16/17

b) Search for Alternatives: Federal law requires that the PI conduct a documented search for alternatives to these procedures. This includes a written narrative describing the written and electronic sources surveyed to identify potential alternatives to painful procedures. Complete the required form at the end of this document.

9. State the rationale for use of this/these species and life stages. Address the issue of replacement by explaining why educational or research objectives cannot be met by the use of nonvertebrate animals, cell or tissue cultures, or non-animal systems. (Please note: the IACUC does not consider "hands-on experience" to be in and of itself an adequate educational objective, unless the course serves students whose anticipated educational and professional futures will require the skills imparted through such hands-on experience. If that is true in this instance, please describe the student population that typically enrolls in the course.)

MPO and fli1 *Danio rerio* are the optimal models for this experiment due to the ease of observation that they allow because of their embryonic transparency, as well as the fact that they provide more complex biological phenomena that more closely resemble those of higher-level organisms than simple cultured cells. The use of later-stage larval and juvenile zebrafish is required to prevent unnecessary embryo fatalities due to the reduced stamina and overall physiological weakness of 96 hpf and younger zebrafish as compared to older fish. Though the innate immune response of zebrafish activates around 30hpf, this experiment will only focus on the observation of 96hpf and older fish because the procedure will be more easily conducted due to the relative size of the model to the implant. Furthermore, the developmental period for the adaptive immune response of zebrafish can range between 4-6 weeks post-fertilization, as described in Question 7, therefore older fish are required for the observation of these interactions. The MPO zebrafish are suitable due to the fact that their neutrophils express GFP, allowing quantification of immune response based on the observation of neutrophil migration. The fli1 zebrafish express GFP throughout their vasculature, providing a quantifiable method for observing implant integration into the host.

10. Justify the number of animals with respect to your overall project design:

a. Study Groups (e.g., treatments and replicates): Briefly outline the specific groups or treatment types that comprise your project. Describe the role each of these groups performs with respect to your specific project objectives/hypotheses (e.g., control or comparison to another treatment). Indicate whether and how these groups would be replicated.

The Class C treatment will consist of juvenile zebrafish that will be euthanized immediately prior to microincision and implantation practice. As this is a pilot study, this treatment will provide a platform for assessing the most effective procedure through which

to proceed when conducting microincisions and implantation, including the possibility of injecting the nanocellulose instead. This treatment will also allow practice for the consistency of both procedures and the respective imaging techniques involved to ensure uniformity between replicates of the main experiment.

Class C Treatment:	Number of Replicates:	Number of Zebrafish:
Euthanasia	30	30
TOTAL:	**30**	**30**

There will be 8 treatment groups under the Class D specification:

Treatment 1: Negative Control, incision (MPO)

Treatment 2: Negative Control, incision (fli1)

Treatment 3: Positive Control, implantation of glass (MPO)

Treatment 4: Positive Control, implantation of glass (fli1)

Treatment 5: Nanocellulose Shard Shape 1 (MPO)

Treatment 6: Nanocellulose Shard Shape 1 (fli1)

Treatment 7: Nanocellulose Shard Shape 2 (fli1)

Treatment 8: Nanocellulose Shard Shape 3 (fli1)

Each treatment will contain 30 replicates, where 1 replicate = 1 fish, and a single shard of the indicated material and shape will be implanted in each replicate. 270 fish will be required for the project. This setup will provide insight into the relationship between the structure of the implant and the cellular integration of the nanocellulose into the tissue of the zebrafish. It will also permit the observation of the effect implant shape may have upon the immune response to the nanocellulose, and may indicate physiological preferences such as porosity for an optimized implant structure. The CNFs that will be utilized are produced with an initial chemical or enzymatic treatment, then mechanically dissociated to create homogenous fibrils. The fibrils are reformed by adding a water solution and then evaporating the excess to form specific densities of material. Within the University of Maine, experiments have previously been conducted with cellular co-cultures to assess any potential residual toxicity the nanocellulose may contain from the initial production step.

Therefore, a similar assay will be performed prior to implantation to ensure no toxic components remain. According to an article by Ning Lin and Alain Dufresne, CNFs have not shown any cytotoxicity or proneness to inflammation in studies on mouse or human macrophages.(9)

The density of the nanocellulose that will be utilized is approximately 1.063g/cm^3, only 6.3% more dense than that of water, and the glass shard contains a density of 2.4mg/cm^3. Therefore, the proposed implants will impart a negligible effect on the ability of the zebrafish to swim. The estimated implant dimensions are: 2.0mm length, 0.025mm depth, and 1.0mm width, with a mass of approximately 53.2µg for the nanocellulose. The glass shard will be formed so that it is comparable in size to the nanocellulose. According to growth studies performed by Hachicho, et.al. and Avella, et.al., the mean wet weight of a 6dpf zebrafish is about 0.4mg, and the mean dry weight of a 96hpf zebrafish is about 70µg.(35,36) As the proposed procedure will be performed on zebrafish 96hpf or later, the nanocellulose implant will be about 76% of its dry weight at 96hpf, and about 13.3% of its wet weight at 6dpf, using the estimates mentioned above. In observation of these ratios, assuming a wet weight value is much more realistic, and allowing a longer development period prior to implantation decreases any negative effects the shard may exert on the behavior of the zebrafish. After implantation, it is expected that the pores of the nanocellulose will be permeated by cellular integration or the extracellular fluid of the fish.

Class D Treatments:	**Number of Replicates:**	**Number of fli1 Zebrafish:**
Incision Only	30	30
Glass Shard	30	30
Nanocellulose Shard, Shape 1	30	30
Nanocellulose Shard, Shape 2	30	30
Nanocellulose Shard, Shape 3	30	30
TOTAL:	**150**	**150**

Class D Treatments:	**Number of Replicates:**	**Number of MPO Zebrafish:**

Incision Only	30	30
Glass Shard	30	30
Nanocellulose Shard, Shape 1	30	30
TOTAL:	**90**	**90**

a. Sample Sizes: Provide a rationale for the number of individuals (per study group or replicate) based on the specific inferential methods to be used. Address the issue of reduction by explaining why the proposed number individuals is sufficient, but not excessive. A simple statement that the number proposed is required for statistical significance is not an adequate response. Formal power analyses often provide the most direct and informative rationale, and are useful in assessing sample sufficiency even when numbers are logistically limited by captures, space etc. See How to do a Power Analysis. If a rationale is based on comparison to prior studies, or specific recommendations for a field, provide relevant citations and justify how the current design compares to those contexts. In the case of pilot studies, meaning investigations conducted for the express purpose of determining suitable approaches and sample sizes for future research, justify your numbers in terms of those objectives.

The sample size for this investigation was based on the experimentation trials for the implantation of elastomer tags conducted by Hohn and Petrie-Hanson, specifically Trials 1-3.[(1)] Those procedures detailed the use of sample sizes of 10 or 30 zebrafish per replicate to ensure proper investigation of a similar surgery, so to reduce the number of animals, this proposed experiment will be utilizing 30 zebrafish for each trial as described in *10.a.* for the observation of immune response and integration. A total of 270 zebrafish will be required, as 30 fish per Class D trial and 30 Class C fish will provide sufficient data to establish a typical immune response between replicates while maintaining a survival margin to account for possible mortalities or infections to ensure quantifiable data. A larger number of fli1 zebrafish will be utilized for the specific comparison of tissue integration between shard shapes, while the MPO fish will be utilized for only one shard shape to assess the immune response toward the CNFs. The aforementioned tagging procedure was performed and monitored through the juvenile stage, which is in agreement with the premise of our implant procedure.

1. Arizona Game and Fish Department. Fish Tagging and Marking Techniques [Internet]. 2013 [cited 2017 May 9]. Available from: http://www.azgfd.gov/w_c/Fish_Tagging_Marking_Techniques.shtml/

2. Sciences B. PIT Tagging : Simple Technology at Its Best. 2017;54(5):447–54.

3. Baras E, Malbrouck C, Houbart M, Kestemont P, Melard C. The effect of PIT tags on growth and physiology of age-0 cultured Eurasian perch Perca flu Õ iatilis of variable size. 2000;

4. Rompen E. The effect of material characteristics , of surface topography and of implant components and connections on soft tissue integration : a literature review. 2006;17:55–67.

5. Bo O, Pihlajama H. Clinical biocompatibility of biodegradable orthopaedic implants for internal " xation : a review. 2000;21.

6. Ambrose CG, Clanton TO. Bioabsorbable Implants : Review of Clinical Experience in Orthopedic Surgery. Ann Biomed Eng. 2006;32(1):171–7.

7. Jorfi M, Foster EJ. Recent advances in nanocellulose for biomedical applications. J Appl Polym Sci. 2015;132(14).

8. Morán JI, Alvarez VA, Cyras VP, Vázquez A. Extraction of cellulose and preparation of nanocellulose from sisal fibers. Cellulose. 2008;15(1):149–59.

9. Lin N, Dufresne A. Nanocellulose in biomedicine: Current status and future prospect. Eur Polym J [Internet]. 2014;59:302–25. Available from: http://www.sciencedirect.com/science/article/pii/S0014305714002493

10. Torres FG, Troncoso OP, Torres C, Grande CJ. Advances in Natural Polymers. Adv Struct Mater [Internet]. 2013;18:21–54. Available from: http://www.scopus.com/inward/record.url?eid=2-s2.0-84874283870&partnerID=tZOtx3y1

11. Abraham E, Deepa B, Pothan LA, Jacob M, Thomas S, Cvelbar U, et al. Extraction of nanocellulose fibrils from lignocellulosic fibres: A novel approach. Carbohydr Polym [Internet]. 2011;86(4):1468–75. Available from: http://dx.doi.org/10.1016/j.carbpol.2011.06.034

12. Prakash P, Kasaby HE, Aggarwal RK, Humfrey S. Microincision bimanual phacoemulsification and Thinoptx implantation through a 1.70 mm incision. Eye (Lond). 2007;21(2):177–82.

13. Fine IH, Hoffman RS, Packer M. Optimizing refractive lens exchange with bimanual microincision phacoemulsification. J Cataract Refract Surg. 2004;30(3):550–4.

14. Ahoor M, Sorkhabi R. Intraocular Foreign Body Removal by 23-Gauge Micro Incision Vitrectomy Surgery and Back Flush Flute Needle: A Case Series Study. Glob J Health Sci [Internet]. 2016;9(5):262. Available from: http://www.ccsenet.org/journal/index.php/gjhs/article/view/56079

15. Sambasivarao S V. OCT-Documented incision features and natural history of clear corneal incisions used for bimanual microincision cataract surgery. Cornea. 2013;18(9):1199–216.

16. Gerlach GF, Morales EE, Wingert RA. Microbead Implantation in the Zebrafish Embryo. J Vis Exp [Internet]. 2015;(101):e52943. Available from: http://www.jove.com/video/52943/microbead-implantation-in-the-zebrafish-embryo

17. Brittijn SA, Duivesteijn SJ, Belmamoune M, Bertens LFM, Bitter W, De Bruijn JD, et al. Zebrafish development and regeneration: New tools for biomedical research. Int J Dev Biol. 2009;53(5–6):835–50.

18. Richardson R, Slanchev K, Kraus C, Knyphausen P, Eming S, Hammerschmidt M. Adult zebrafish as a model system for cutaneous wound-healing research. J Invest Dermatol [Internet]. 2013;133(6):1655–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23325040%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3644348

19. Chang W, Hwang P. Development of Zebrafish Epidermis. 2011;214(Part C):205–14.

20. Sullivan C, Kim CH. Zebrafish as a model for infectious disease and immune function. Fish Shellfish Immunol. 2008;25(4):341–50.

21. Lambris JD. Current Topics in Innate Immunity II.

22. Mathias JR, Perrin BJ, Liu T, Kanki J, Look AT, Huttenlocher A. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish flammation is a critical process during normal im-. 2006;80(December).

23. Rosales C, Demaurex N, Lowell CA, Uribe-querol E. Neutrophils : Their Role in Innate and Adaptive Immunity. 2016;2016:2–4.

24. Developmental stage data for this paper were retrieved from the Zebrafish Information Network (ZFIN); University of Oregon; Eugene; OR 97403-5274; URL: http://zfin.org/; 9/20/17.

25. Renshaw S, Loynes C. A transgenic zebrafish model of neutrophilic inflammation. Blood… [Internet]. 2006;108(13):3976–8. Available from: http://bloodjournal.hematologylibrary.org/content/108/13/3976.short

26. Lawson ND, Weinstein BM. In Vivo Imaging of Embryonic Vascular Development Using Transgenic Zebrafish. Dev Biol [Internet]. 2002;248(2):307–18. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0012160602907116

27. White RM, Sessa A, Burke C, Bowman T, Leblanc J, Ceol C, et al. Resource

Transparent Adult Zebrafish as a Tool for In Vivo Transplantation Analysis. 2008;(February):183–9.

28. Karlsson J, von Hofsten J, Olsson P-E. Generating Transparent Zebrafish: A Refined Method to Improve Detection of Gene Expression During Embryonic Development. Mar Biotechnol [Internet]. 2001;3(6):0522–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14961324%5Cnhttp://link.springer.com/10.1007/s1012601-0053-4

29. Tobias CW, Eisenberg M. IN ELECTROLYSIS-- REVIEW. 99(12):359–65.

30. Moore SW, Kennedy TE. Dissection and culture of embryonic spinal commissural neurons. Curr Protoc Neurosci. 2008;(SUPPL. 43).

31. Conrad GW, Bee JA, Roche SM, Teillet MA. Fabrication of microscalpels by electrolysis of tungsten wire in a meniscus. J Neurosci Methods. 1993;50(1):123–7.

32. Fang P, Lin JF, Pan HC, Shen YQ, Schachner M. A Surgery Protocol for Adult Zebrafish Spinal Cord Injury. J Genet Genomics [Internet]. 2012;39(9):481–7. Available from: http://dx.doi.org/10.1016/j.jgg.2012.07.010

33. OLYMPUS. Research Inverted System Microscope Ix71/Ix81 Ix2 Series. In: Manual.

34. Wikki Stix One of a Kind Creatables [Internet]. Available from: https://www.wikkistix.com/

35. Hachicho N, Reithel S, Miltner A, Heipieper HJ, Küster E, Luckenbach T. Body mass parameters, lipid profiles and protein contents of zebrafish embryos and effects of 2,4-dinitrophenol exposure. PLoS One. 2015;10(8):1–19.

36. Avella MA, Place A, Du SJ, Williams E, Silvi S, Zohar Y, et al. Lactobacillus rhamnosus Accelerates Zebrafish Backbone Calcification and Gonadal Differentiation through Effects on the GnRH and IGF Systems. PLoS One. 2012;7(9).

37. Hohn C, Petrie-Hanson L. Evaluation of visible implant elastomer tags in zebrafish (Danio rerio). Biol Open [Internet]. 2013;2(12):1397–401. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3863425&tool=pmcentrez&rendertype=abstract

38. Peng S, Yao L, Cui C, Zhao H, Liu C, Li Y, et al. Semaphorin4D promotes axon regrowth and swimming ability during recovery following zebrafish spinal cord injury. Neuroscience [Internet]. 2017;351:36–46. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0306452217301963

39. Collymore C, Tolwani A, Lieggi C, Rasmussen S. Efficacy and safety of 5

anesthetics in adult zebrafish (Danio rerio). J Am Assoc Lab Anim Sci. 2014;53(2):198–203.

40. Huang W-C, Hsieh Y-S, Chen I-H, Wang C-H, Chang H-W, Yang C-C, et al. Combined Use of MS-222 (Tricaine) and Isoflurane Extends Anesthesia Time and Minimizes Cardiac Rhythm Side Effects in Adult Zebrafish. Zebrafish [Internet]. 2010;7(3):297–304. Available from: http://www.liebertonline.com/doi/abs/10.1089/zeb.2010.0653

c. Summary: Provide summary formula(s) that clearly depict how the numbers of individuals listed in #8 above are obtained as a product of the number of study groups, replicates and sample sizes presented in 10a and 10b.

Class C: 30 juvenile zebrafish = 1 exposure group * 30 individuals per group * 1 age point

Class D (fli1): 150 juvenile fli1zebrafish = 5 exposure groups (including controls) * 30 individuals per group * 1 age point

Class D (MPO): 90 juvenile MPO zebrafish = 3 exposure groups (including controls) * 30 individuals per group * 1 age point

11. Procedures

The Committee does not wish to receive copies of research proposals or laboratory manuals. The Principal Investigator or Instructor is asked to address succinctly the following questions, as applicable. Special care should be taken to justify any procedures generally discouraged by the University's code of ethics and policy.

a. Major categories of procedures. Please check the appropriate box for **EACH category**.

Any "yes" responses must be described in sections b. (nonsurgical procedures), c. (surgical procedures) or d. (euthanasia) that follow.

Yes	No	Categories

☐ ☒ 1. collection or capture (provide details under section 12)

☐ ☒ 2. nonsurgical marking, tagging, or device attachment

☐ ☒ 3. antibody production: describe antigen, adjuvant and route of immunization

☒ ☐ 4. noninvasive physical or physiological measurements

☐ ☒ 5. dietary manipulations

☒ ☐ 6. pharmacology/toxicology: material used, route of administration, etc.

☐ ☒ 7. blood draw, biopsy or other nonsurgical tissue collection

☐ ☒ 8. behavior studies

☐ ☒ 9. environmental stress, e.g., temperature, restraint, forced exercise

☐ ☒ 10. irradiation: type, facility to be used

☐ ☒ 11. hazardous materials, e.g., carcinogens, radioactive materials

☐ ☒ 12. biohazardous or infectious agents (use of Class 2 or higher agents requires the approval of the University's Biosafety Committee). Description must include precautions to restrict the spread of biohazardous or infectious agents to non-target animals or humans.

☐ ☒ 13. experimental trauma, e.g., planned injury, significant behavioral stress

☒ ☐ 14. anesthesia/sedation/immobilization (describe in sections 11 b or 11 c)

☐ ☒ 15. nonsurvival surgical procedure

☒ ☐ 16. survival surgical procedure (animal is allowed to recover for any length of time)

☐ ☒ 17. multiple major operative procedures from which animal is allowed to recover

☐ ☒ 18. planned euthanasia (describe method in section 11 d, e.g., harvest tissue, necropsy, etc.)

☐ ☒ 19. other, specify:

b. Nonsurgical Procedures (**Categories 2-14 and potentially 19**):

1. **USING THE ABOVE NONSURGICAL CATEGORIES MARKED "YES" AS HEADINGS**, provide a succinct description of the procedures to be conducted on live vertebrate animals. Specify any drug(s), including adjuvants, doses (including frequency) and routes of administration. Specify duration of procedures. Include any monitoring procedures used to ensure effective anesthesia/sedation or recovery from other nonsurgical procedures.

4. Noninvasive physical or physiological measurements: After 3 days post-operation, fish will be immobilized with MS-222 (200 mg Tris-buffered tricane methanesulfonate/liter), then imaged with fluorescence microscopy and released immediately; procedure should take 2-3 minutes.

6. Pharmacology/toxicology: At 24 hpf, embryos will be subjected to a continuous treatment of 1-phenyl 2-thiourea (PTU) at a concentration of 11.4mg/L in an egg water solution. This treatment will persist for the duration of the experiment, where the solution will be changed daily, according to Karlsson, Hofston, and Olsson.(28)

6. Pharmacology/toxicology: Cephalexin and methylene blue will be used as antibiotic and antifungal reagents, respectively, directly after the surgery has been performed. 6.6mg/L of cephalexin and 30mg/L of methylene blue will be added to the holding tank for 3 days post-operation, where the solution will be changed daily.

14. Anesthesia/sedation/immobilization: Fish will be placed in a solution of MS-222 (200 mg Tris-buffered tricane methanesulfonate/liter) for up to 45 seconds for immobilization during implantation and imaging.

c. Surgical Procedures (**Items 14-17; and potentially 19**):

1. **USING THE ABOVE SURGICAL CATEGORIES MARKED "YES" AS HEADINGS**, provide a succinct description of the surgical procedures to be conducted on live vertebrate animals. Specify any drug(s), including adjuvants, doses (including frequency) and routes of administration. Specify duration of procedures. Be sure to include any monitoring procedures use to ensure safe and effective anesthesia/sedation.

16. Survival surgical procedure:

Following anesthesia, an incision of approximately 1mm will be made along the dorsal line caudal to the dorsal fin using electrolytically-sharpened tungsten wire, and a shard of solid-form nanocellulose will be inserted. The incision will be intramuscular only, and the implant will be completely subcutaneous for three-dimensional cellular integration. As an alternative that will be determined by the Class C trials, the implant may be injected using standard tuberculin syringes, in which case the injury could be further reduced.

The nanocellulose shards, tungsten wire, and/or the syringe will have been sterilized in a solution of 95% ethanol and left to dry under the fume hood prior to the surgery. The tungsten wire will be sterilized in 95% ethanol for 30 seconds between each fish procedure. Procedure should take up to 1 minute per fish, and incision size is refined to minimize affected tissue to reduce the potential of residual pain. No sealant will be used on the incision post-implantation as zebrafish skin re-epithelializes within a few hours, reducing the chance of environmental contamination.(18) Furthermore, a comparison with the negative control and the implant treatments will aid in determining the possibility of contamination.

2. Is animal allowed to regain consciousness after surgery?

Yes.

3. Describe the postsurgical monitoring and care procedures, including what response(s) you will look for to indicate recovery or deterioration. Indicate dosage or frequency of any analgesics, other drugs, or pain relieving measures that will be used post-operatively.

Postsurgical monitoring will include regular tank maintenance and upkeep for the duration of the experiment where behavior/physical appearance of the zebrafish will be

observed once daily until feeding age (120 hpf) has been reached, then twice daily for the remainder of the experiment, including the post-surgery timeframe of one month. Dead fish will be removed to prevent contamination. Upon immediate completion of the surgery, stress experienced by the fish will be managed by placing the fish in a dish of egg water to regain consciousness, then gently reintroducing the fish to their holding tank to allow acclimatization. The holding tank will contain a solution of cephalexin (6.6mg/L) and methylene blue (30mg/L) to prevent infection that could interfere alter the response to the implant. The zebrafish will be maintained in this antibiotic solution for 3 days post-surgery following the protocol by Fang, et. al, then returned to normal holding conditions thereafter.(32)

The physical movement of the fish will be observed post-operation for up to 10 minutes to ensure that normal behavior is restored. If severe distress is evident from unusual behavior (i.e. prolonged erratic swimming, persistent color change indicating stress, etc.), the affected fish will be removed from the experiment and promptly euthanized as described in *11.d.3.* to avoid extended distress. Since the surgical procedure is performed only once during the initial portion of the experiment, no other analgesics will be administered after the zebrafish have regained consciousness to prevent unnecessary deleterious side-effects, such as weakened cardiovascular strength that may lead to death.

d. Euthanasia (Category 18 and unplanned euthanasia):

1. Will the animals be killed as part of the study design or at the conclusion of the study?

Yes, at the conclusion of the study.

2.* If yes, how will this be accomplished (include dosages/duration if applicable) and verified?

The fish will be euthanized with sodium bicarbonate-buffered MS-222 once the study has gone to completion. The zebrafish research community has prepared its own detailed user manual that describes the standard procedures used by zebrafish researchers. The zebrafish standard operating procedure manual is The Zebrafish Book (1995). The Zebrafish Book covers all of the experimental procedures proposed and is available on the

internet: go to http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg and choose the "The Zebrafish Book" under “News and Information” on the sidebar menu. This procedure is planned to be performed only when the experiment is concluded.

The animals will be euthanized with an overdose of sodium bicarbonate- buffered MS222 (300 mg/L) for a duration of 10 minutes or until the heart has stopped beating.

3.* THIS QUESTION MUST BE ANSWERED: If euthanasia becomes necessary due to unplanned injury or illness to the animal(s), how will it be accomplished (include dosages/duration if applicable) and verified?

If the animals become injured or sustain an illness, they will be euthanized with an overdose of sodium bicarbonate-buffered MS222 (300 mg/L) for a duration of 10 minutes or until the heart has stopped beating, as above.

*See the 2013 Report of the AVMA Panel on Euthanasia for assistance (https://www.avma.org/KB/Policies/Documents/euthanasia.pdf). NOTE: When possible, euthanasia should be conducted in a place or fashion that minimizes the potential for cues that could cause distress in other animals (e.g., outside housing room or in an isolated chamber or container).

12. Animal Sources and Housing

a. Please indicate source of animals. Note: The IACUC will approve animal purchases from a licensed pet store provided the researcher/instructor informs the pet store (in writing) that the purchased animals will be used for research/teaching.

☒ Purchased or conveyed from a company or other institution

(please answer the following)

1. What are the specific planned commercial or institutional sources?

   Zebrafish embryos will be obtained from natural spawnings of adult zebrafish in the UMaine fish facility.

☐ Captured from the wild (please answer the following)

1. Where and when will the animals be captured?

2. What specific capture gear will be employed (nets, traps, electrofishing etc.) and how will it be operated (e.g., frequency of net or trap checks)

3. What steps will be taken to protect animals from exposure or other danger during collection?

4. Please include your plans for removal of traps, barriers or other gear from the field site.

5. Indicate if Federal/State permits are required and whether they have been obtained. **NOTE: Permit documents must be made available if requested by the IACUC.**

6. What precautions will be taken in the field to restrict the spread of pathogens among study animals or between study animals and humans?

b. Animal Care/Housing:

IMPORTANT NOTE: Investigators are expected to follow care and housing guidelines outlined in the *Guide for the Care and Use of Lab Animals* (http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf) or the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (http://www.fass.org/docs/agguide3rd/Ag_Guide_3rd_ed.pdf) unless special exceptions are requested and approved. If specific requirements for your animals are not listed in the Guides (e.g. some wildlife), you are expected to adhere to recommended practices of the field (e.g., as outlined by professional societies) and known biological needs of the species. All investigators working with housed animals are expected to keep records of daily care/feeding, as well as records of other periodic care (e.g., grooming, water quality) for inspection by the IACUC.

Will animals be housed or maintained for more than 12 hours?

☒ Yes

☐ No

If yes – answer the following

1. Where will the animals be housed and maintained?

They will be housed and maintained in Room 181, Hitchner Hall in the secondary zebrafish facility at the University of Maine for the duration of the experiment.

2. Does your housing deviate from the requirements of the Guides or recommended practices? If so, include a justification for an exception to the Guides for taxa covered therein. For taxa not covered by the guides, specify any other guidelines you intend to follow, or provide a detailed description of housing and care based on your study organism's known requirements.

No.

3. For genetically modified animals (GMAs – produced via targeted or random genetic manipulations), the Guide requires enhanced monitoring and reporting to the IACUC. If this protocol involves GMAs, describe any special care and monitoring (including frequency) that will be used to minimize known or unknown adverse effects in the genetically altered line.

We will be using the MPO *Danio rerio*, a transgenic line that expresses GFP on a neutrophil-specific myeloperoxidase promoter, where EGFP with an SV40 polyadenylation site was inserted at the mpo ATG start site.(25) We will also be using the transgenic line, fli1 *Danio rerio,* that expresses EGFP along its vasculature through the use of a promoter for *fli1*.(26) These transgenic lines are both similar to wildtype zebrafish. The MPO line was developed specifically for use in immune response studies by Renshaw, et.al. at the MRC Centre for Developmental and Biomedical Genetics, University of Sheffield, Sheffield UK to provide a more efficient research method for imaging neutrophil migration. The fli1 line was developed for observation of blood vessel development in zebrafish by Lawson and Weinstein at the Laboratory of Molecular Genetics, NICHD, NIH in Bethesda, Maryland.(26) These lines are used throughout the zebrafish community and typically require no special care or monitoring; however, for these experiments, animals will be examined daily and water will be changed daily to ensure humane treatment. Adult fish have been present in the zebrafish facility for years and are under the direct care and supervision of the facility manager.

1. Arizona Game and Fish Department. Fish Tagging and Marking Techniques [Internet]. 2013 [cited 2017 May 9]. Available from: http://www.azgfd.gov/w_c/Fish_Tagging_Marking_Techniques.shtml/

2. Sciences B. PIT Tagging : Simple Technology at Its Best. 2017;54(5):447–54.

3. Baras E, Malbrouck C, Houbart M, Kestemont P, Melard C. The effect of PIT tags on growth and physiology of age-0 cultured Eurasian perch Perca flu Õ iatilis of variable size. 2000;

4. Rompen E. The effect of material characteristics , of surface topography and of implant components and connections on soft tissue integration : a literature review. 2006;17:55–67.

5. Bo O, Pihlajama H. Clinical biocompatibility of biodegradable orthopaedic implants for internal " xation : a review. 2000;21.

6. Ambrose CG, Clanton TO. Bioabsorbable Implants : Review of Clinical Experience in Orthopedic Surgery. Ann Biomed Eng. 2006;32(1):171–7.

7. Jorfi M, Foster EJ. Recent advances in nanocellulose for biomedical applications. J Appl Polym Sci. 2015;132(14).

8. Morán JI, Alvarez VA, Cyras VP, Vázquez A. Extraction of cellulose and preparation of nanocellulose from sisal fibers. Cellulose. 2008;15(1):149–59.

9. Lin N, Dufresne A. Nanocellulose in biomedicine: Current status and future prospect. Eur Polym J [Internet]. 2014;59:302–25. Available from: http://www.sciencedirect.com/science/article/pii/S0014305714002493

10. Torres FG, Troncoso OP, Torres C, Grande CJ. Advances in Natural Polymers. Adv Struct Mater [Internet]. 2013;18:21–54. Available from: http://www.scopus.com/inward/record.url?eid=2-s2.0-84874283870&partnerID=tZOtx3y1

11. Abraham E, Deepa B, Pothan LA, Jacob M, Thomas S, Cvelbar U, et al. Extraction of nanocellulose fibrils from lignocellulosic fibres: A novel approach. Carbohydr Polym [Internet]. 2011;86(4):1468–75. Available from: http://dx.doi.org/10.1016/j.carbpol.2011.06.034

12. Prakash P, Kasaby HE, Aggarwal RK, Humfrey S. Microincision bimanual phacoemulsification and Thinoptx implantation through a 1.70 mm incision. Eye (Lond). 2007;21(2):177–82.

13. Fine IH, Hoffman RS, Packer M. Optimizing refractive lens exchange with bimanual microincision phacoemulsification. J Cataract Refract Surg. 2004;30(3):550–4.

14. Ahoor M, Sorkhabi R. Intraocular Foreign Body Removal by 23-Gauge Micro Incision Vitrectomy Surgery and Back Flush Flute Needle: A Case Series Study. Glob J Health Sci [Internet]. 2016;9(5):262. Available from: http://www.ccsenet.org/journal/index.php/gjhs/article/view/56079

15. Sambasivarao S V. OCT-Documented incision features and natural history of clear corneal incisions used for bimanual microincision cataract surgery. Cornea. 2013;18(9):1199–216.

16. Gerlach GF, Morales EE, Wingert RA. Microbead Implantation in the Zebrafish

Embryo. J Vis Exp [Internet]. 2015;(101):e52943. Available from: http://www.jove.com/video/52943/microbead-implantation-in-the-zebrafish-embryo

17. Brittijn SA, Duivesteijn SJ, Belmamoune M, Bertens LFM, Bitter W, De Bruijn JD, et al. Zebrafish development and regeneration: New tools for biomedical research. Int J Dev Biol. 2009;53(5–6):835–50.

18. Richardson R, Slanchev K, Kraus C, Knyphausen P, Eming S, Hammerschmidt M. Adult zebrafish as a model system for cutaneous wound-healing research. J Invest Dermatol [Internet]. 2013;133(6):1655–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23325040%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3644348

19. Chang W, Hwang P. Development of Zebrafish Epidermis. 2011;214(Part C):205–14.

20. Sullivan C, Kim CH. Zebrafish as a model for infectious disease and immune function. Fish Shellfish Immunol. 2008;25(4):341–50.

21. Lambris JD. Current Topics in Innate Immunity II.

22. Mathias JR, Perrin BJ, Liu T, Kanki J, Look AT, Huttenlocher A. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish flammation is a critical process during normal im-. 2006;80(December).

23. Rosales C, Demaurex N, Lowell CA, Uribe-querol E. Neutrophils : Their Role in Innate and Adaptive Immunity. 2016;2016:2–4.

24. Developmental stage data for this paper were retrieved from the Zebrafish Information Network (ZFIN); University of Oregon; Eugene; OR 97403-5274; URL: http://zfin.org/; 9/20/17.

25. Renshaw S, Loynes C. A transgenic zebrafish model of neutrophilic inflammation. Blood… [Internet]. 2006;108(13):3976–8. Available from: http://bloodjournal.hematologylibrary.org/content/108/13/3976.short

26. Lawson ND, Weinstein BM. In Vivo Imaging of Embryonic Vascular Development Using Transgenic Zebrafish. Dev Biol [Internet]. 2002;248(2):307–18. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0012160602907116

27. White RM, Sessa A, Burke C, Bowman T, Leblanc J, Ceol C, et al. Resource Transparent Adult Zebrafish as a Tool for In Vivo Transplantation Analysis. 2008;(February):183–9.

28. Karlsson J, von Hofsten J, Olsson P-E. Generating Transparent Zebrafish: A Refined Method to Improve Detection of Gene Expression During Embryonic

Development. Mar Biotechnol [Internet]. 2001;3(6):0522–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/14961324%5Cnhttp://link.springer.com/10.1007/s1012601-0053-4

29. Tobias CW, Eisenberg M. IN ELECTROLYSIS-- REVIEW. 99(12):359–65.

30. Moore SW, Kennedy TE. Dissection and culture of embryonic spinal commissural neurons. Curr Protoc Neurosci. 2008;(SUPPL. 43).

31. Conrad GW, Bee JA, Roche SM, Teillet MA. Fabrication of microscalpels by electrolysis of tungsten wire in a meniscus. J Neurosci Methods. 1993;50(1):123–7.

32. Fang P, Lin JF, Pan HC, Shen YQ, Schachner M. A Surgery Protocol for Adult Zebrafish Spinal Cord Injury. J Genet Genomics [Internet]. 2012;39(9):481–7. Available from: http://dx.doi.org/10.1016/j.jgg.2012.07.010

33. OLYMPUS. Research Inverted System Microscope Ix71/Ix81 Ix2 Series. In: Manual.

34. Wikki Stix One of a Kind Creatables [Internet]. Available from: https://www.wikkistix.com/

35. Hachicho N, Reithel S, Miltner A, Heipieper HJ, Küster E, Luckenbach T. Body mass parameters, lipid profiles and protein contents of zebrafish embryos and effects of 2,4-dinitrophenol exposure. PLoS One. 2015;10(8):1–19.

36. Avella MA, Place A, Du SJ, Williams E, Silvi S, Zohar Y, et al. Lactobacillus rhamnosus Accelerates Zebrafish Backbone Calcification and Gonadal Differentiation through Effects on the GnRH and IGF Systems. PLoS One. 2012;7(9).

37. Hohn C, Petrie-Hanson L. Evaluation of visible implant elastomer tags in zebrafish (Danio rerio). Biol Open [Internet]. 2013;2(12):1397–401. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3863425&tool=pmcentrez&rendertype=abstract

38. Peng S, Yao L, Cui C, Zhao H, Liu C, Li Y, et al. Semaphorin4D promotes axon regrowth and swimming ability during recovery following zebrafish spinal cord injury. Neuroscience [Internet]. 2017;351:36–46. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0306452217301963

39. Collymore C, Tolwani A, Lieggi C, Rasmussen S. Efficacy and safety of 5 anesthetics in adult zebrafish (Danio rerio). J Am Assoc Lab Anim Sci. 2014;53(2):198–203.

40. Huang W-C, Hsieh Y-S, Chen I-H, Wang C-H, Chang H-W, Yang C-C, et al. Combined Use of MS-222 (Tricaine) and Isoflurane Extends Anesthesia Time and

Minimizes Cardiac Rhythm Side Effects in Adult Zebrafish. Zebrafish [Internet]. 2010;7(3):297–304. Available from: http://www.liebertonline.com/doi/abs/10.1089/zeb.2010.0653

4. Identify the room or facility in which the procedures will be conducted.

All procedures will be conducted in Hitchner Hall.

5. What precautions will be taken to restrict the inadvertent spread of pathogens among study animals or between study animals and humans?

Proper PPE procedures will be followed (gloves), and all surfaces will be wiped down with ethanol after handling is concluded.

13. List all person(s) (including PI) who will handle animals (e.g., carry out the procedure(s), animal care, etc.) or provide training of personnel. For each person named below, describe his/her individual experience in performing proposed procedures (e.g., years of experience and specific skills); if none, explain how training will be obtained. (NOTE: to add rows, right-click within table, click on "insert" and choose "insert rows above" or "insert rows below")

Personnel Name	**Role**	**Years of experience**	**Training plan (if no experience)**
Dr. Paul Millard	PI	8 years with zebrafish (general)	
Dr. Con Sullivan	Assistant Research Professor	12 years with zebrafish (Innate immunity, *TNFAIP8* gene family,	

		tumorigenesis, tumor angiogenesis and inflammation)	
Mark Nilan	Operations Manager	Zebrafish maintenance and husbandry	
Hanna Anderson	Student Research Assistant	2 years with zebrafish (microinjections, dechorionation, general)	

14. If animals will be housed, please list the name, phone number, and email of the person who should be contacted to accompany the IACUC during facility inspections:

Mark Nilan, 581-3391, Mark.Nilan@umit.maine.edu

15. Have all personnel named above been certified by the IACUC for Responsible Care and Use of Animals?

☒ Yes ☐ No A web-based tutorial for this certification is available at: http://umaine.edu/research/research-compliance/institutional-animal-care-and-use-committee-iacuc/web-based-training/. (Note: protocol approval will not be granted until all personnel have been certified.)

If this is a teaching protocol where students will handle animals as part of course participation, please see "Training Requirement for Students Who Handle Live Vertebrate Animals in Class."

Indicate which option you will require your class to follow to meet the training requirement:

☐ Students will complete the web-based tutorial (referenced above).

☐ Students will read the document, "Use of Animals in a Courses: What You Need to Know" (found on the IACUC website under "Training Requirement for Students…" as referenced above).

☐ Students will be trained by the instructor; attached is a written description of the training for IACUC review.

16. Disaster Planning and Emergency Preparedness. The Guide requires that facilities have disaster plans to "define the actions necessary to prevent animal pain, distress, and deaths due to loss of systems such as those that control ventilation, cooling, heating, or provision of potable water." Safety and Environmental Management, in conjunction with the IACUC and researchers, are putting disaster plans in place that meet University and individual investigator needs, include provisions for triage and euthanasia, and provide for training and contact of essential personnel. Please provide the following information:

a. Triage: Some animals may require priority care (or euthanasia) under a facility-wide or campus-wide disaster. For example, they may have greater potential to experience severe pain or distress under disruption of services (e.g., post-operative individuals) or they may be irreplaceable in a replicate study (e.g., novel genetic lines). Do any animals used in this study require special priority for triage? If so, please describe the basis for this special priority and indicate how such animals will be made identifiable within the facility (e.g., special marks, lists).

Yes. The animals used in this study will require special priority for triage in the event of a facility-wide or campus-wide disaster. The animals in this study will be clearly marked with red tape or red dots and labeled as requiring special priority for triage.

b. Special euthanasia: Would a different method of euthanasia than that listed in section 11.d. be used in the event of a disaster that disrupts normal services required for humane care and treatment of these animals? If yes, please describe the special method (include dosing information for pharmaceutical approaches).

No.

c) Satellite Facility: If the facility listed under section 12.b.1 is not a "core" facility (Aquaculture Research Center, Center for Cooperative Aquaculture Research, Small Animal Research Facility, or the Witter Center), the facility must have an approved Satellite Facility Designation and Disaster Plan (contact the IACUC Office for the form).

☐ The facility under section 12.b.1 is designated as a 'core' facility.

☒ A Satellite Facility Designation and Disaster Plan has been approved for this facility.

☐ I have attached a completed Satellite Facility Designation and Disaster Plan for approval.

d. Emergency Contact for the Care of Animals: (at least two people must be listed):

Primary person to contact in case of an emergency = Dr. Paul Millard

Office phone = *207-581-2265*

Home phone = 207-659-9425

Cell phone = 207-659-9425

Secondary person to contact = Dr. Con Sullivan

Office phone =207-581-2809

Home phone =207-884-4911
Cell phone = 207-217-7689

Risk Assessment

(Risks to researchers)

In compliance with our Public Health Service Animal Welfare Assurance, we have implemented an Occupational Health/Medical Surveillance Program. The first step will be for investigators to identify potential hazards with tasks involved with the study, so the IACUC veterinarian and Safety and Environmental Management (SEM) can assess the risks to determine if further information will be required from everyone named in the protocol (i.e., a health questionnaire). **NOTE:** In evaluating this risk assessment statement, we will be looking for animal care tasks that increase the risk of illness (such as a zoonotic disease), physical injury (such as animal bites), and/or allergic reactions to those handling the animals. Also consider hazards of animal excrement/hazards to workers handling the animals' bedding that may be important to an accurate risk assessment.

Please complete the following for your proposed protocol.

NOTE: For field studies, the Field Research Hazard Assessment/Safety Plan will be helpful in identifying possible risks)

a) Provide a brief description of the protocol (cut and paste response from question 6 of the protocol). (NOTE: Only this page, not the whole protocol, goes to SEM and the Occupational Health Physician, thus the request for duplication of the answer to question 6.)

The main objective of this research project is to utilize the zebrafish animal model system to assess biomaterials with potential for use in medical implants. The biomaterial in question is dense nanocellulose in solid form created in the Chemical and Biological Engineering Department facilities at the University of Maine. One aspect of the project will focus on methods for implanting the novel nanocellulose material into zebrafish to evaluate the potential of this material for use in prosthetics in humans, as zebrafish contain a similar. This project holds considerable potential for the advancement of naturally-sourced implantable biomaterials due to the biocompatibility, durability, and availability of nanocellulose. With a porous composition, nanocellulose enables ample cellular integration, permitting a more secure implant. Variations in structure, including the density and specific shape of the nanocellulose shard, affect the integration into tissues, therefore optimization of these parameters is crucial for the improvement of existing implant formats. Implantation will likely require micro-incisions to be made on the model, and/or physical modification of the nanocellulose to permit insertion. A new approach for creating micro-incisions in the zebrafish may need to be developed, as zebrafish are delicate and are only a few millimeters in length, rendering conventional techniques difficult to implement while maintaining the survival of the animal.

When implantation has been accomplished, immune responses and other changes in the condition of surrounding tissue will be monitored and recorded by imaging, including bright-field and fluorescence microscopy. GFP-tagged zebrafish will be utilized for the visualization and quantification of neutrophils migrating to the site of implantation. The amount of neutrophils that migrate will indicate whether the nanocellulose has been rejected or accepted by the model, providing insight into whether it remains inert when introduced to tissue. The observation of cellular integration of the nanocellulose in the model will also be conducted through the use of fli1 zebrafish (GFP-labeled vasculature), as well as the microscopy methods listed above.

b) List the tasks required. (Examples: handling animals, administering drugs, euthanasia; field work could include driving, operating watercraft.)

1. Continuous administration of 1-phenyl 2-thiourea (PTU) in holding tanks
2. Obtainment of nanocellulose shards
3. Sterilization of shards and tungsten wire in 95% ethanol
4. Performing microincisions on zebrafish
5. Implantation of nanocellulose into model
6. Using cephalexin monohydrate (capsule form) and methylene blue (capsule form) for post-surgical treatment of fish
7. Monitor fish through confocal and fluorescence microscopy

c) For each of the tasks described in b) above, list the associated hazards. (Examples; exposure to allergens, needle stick.)

1. Fatal if swallowed, may cause allergic skin reaction

2. Accidental puncture from shards, respiratory exposure

3. Exposure to ethanol, accidental puncture from shards or tungsten wire, respiratory

exposure

4. Sharpened tungsten wire, accidental puncture

5. Accidental puncture from shards, respiratory exposure

6. Cephalexin monohydrate: May cause an allergic skin reaction, may cause allergy or

asthma symptoms or breathing difficulties if inhaled; Methylene blue: ingestion

7. Exposure to fixatives, methanol, and other chemicals; laser

d) For each of the hazards described in c) above list how the hazards will be managed. (Examples: use of gloves and goggles, field work training.)

1. Appropriate PPE (gloves, safety goggles, lab coat) and disposal of materials (biohazard bags); avoid breathing in, contaminated work clothing should not be allowed out of the workplace; Wash face, hands and any exposed skin thoroughly after handling, do not eat, drink or smoke when using.
2. Work in cell culture hood. Appropriate PPE (gloves, safety goggles, lab coat) and disposal of materials (biohazard bags); treat area as standard for puncture injuries (wash with soap and water and watch for signs of infection), work under fume hood to meet exposure limits: OSHA permissible exposure limit (PEL) 15mg/m^3 total dust, 5 mg/m^3 respirable fraction for nuisance dusts.

3. Appropriate PPE (gloves, safety goggles, lab coat) and disposal of materials (biohazard bags); avoid inhalation or eye contact with ethanol; treat area as standard for puncture injuries (wash with soap and water and watch for signs of infection), work under fume hood to meet exposure limits: OSHA permissible exposure limit (PEL) 15mg/m^3 total dust, 5 mg/m^3 respirable fraction for nuisance dusts.

4. Appropriate PPE (gloves, safety goggles, lab coat) and disposal of materials (biohazard bags); treat area as standard for puncture injuries (wash with soap and water and watch for signs of infection)

5. Appropriate PPE (gloves, safety goggles, lab coat) and disposal of materials (biohazard bags); treat area as standard for puncture injuries (wash with soap and water and watch for signs of infection), work under fume hood to meet exposure limits: OSHA permissible exposure limit (PEL) 15mg/m^3 total dust, 5 mg/m^3 respirable fraction for nuisance dusts.

6. Appropriate PPE (gloves, safety goggles, lab coat) and disposal of materials (biohazard

bags); Use outdoors or in a well-ventilated area, modify administration method by opening the capsule underwater to prevent airborne material; Wash face, hands and any exposed skin thoroughly after handling, do not eat, drink or smoke when using.

7. Appropriate PPE (gloves, safety goggles, lab coat) and disposal of materials (biohazard bags); Restricted access with appropriate signage. Avoid direct eye contact.

After this risk assessment is reviewed, everyone named in the protocol may be required to complete a health questionnaire. The health questionnaire may require review by the Occupational Health Physician. If so, there is a charge for this review (~$45). Researchers are asked to budget for these costs in proposals for outside funding. For unfunded studies, the cost will be covered by the Office of the Vice President for Research. If you have any questions regarding the completion of this page, please contact, Safety and Environmental Management (SEM), 1-4055.

SEARCH FOR ALTERNATIVES TO PAINFUL/DISTRESSFUL PROCEDURES

This form must be completed if the pain classification from Question #8 was D or E

Please read the background information on the USDA policy for painful and distressful procedures before completing this form (Appendix 1).

The written narrative should include adequate information for the IACUC to assess that a reasonable and good faith effort was made to determine the availability of alternatives or alternative methods.

The following information is required:

1) The names(s) of the database(s) searched (due to the variation in subject coverage and sources used, one database is seldom adequate);

ALTBIB

PubMed

ScienceDirect (Elsevier)

Google Scholar

2) The date the search was performed.

4/13/2017

3) The time period covered by the search.

2000-2017

4) The search strategy (including scientifically relevant terminology) used.

A terminology search was conducted across the databases listed above, including the terms: zebrafish incision method, zebrafish implant, zebrafish surgery, zebrafish tissue injury, zebrafish immune response, analgesics zebrafish, *Danio rerio* incision, *Danio rerio* implant.

5) Did your database search (or other source) identify a bona fide alternative method (one that could be used to accomplish the goals of the animal use proposed)?

No.

If yes, please explain why the alternative found was not proposed. (NOTE: The IACUC will consider this explanation, but may determine it is not adequate to justify not using the bona fide alternative.

If no, the IACUC would like a description of the results of the database search (or other source) to document the lack of relevant alternatives.

This database search produced minimal results for implantation studies of this exact nature, but a combination of related investigations utilizing zebrafish yielded methods of comparable pain management. When delving into methods of more serious operations with alternative target results, the anesthesia procedures were similar to that which is proposed here: immersion in a MS-222 solution (200 mg Tris-buffered tricaine methanesulfonate/liter). For example, Richardson, et. al. conducted a study to assess the immune response of zebrafish when a tissue wound was inflicted, and examination of the procedure indicated the use of a 0.13% tricaine solution (MS-222) for the anesthetic, as well as the addition of a hydrocortisone or sodium warfarin treatment to the tank water prior to the operation.(18) The injury involved in this citation was created for the

observation of the regeneration of epithelial cells and the inflammation response at the site of affectation, therefore a large excision was created in the zebrafish tissue. A dermal laser was employed, as well as multiple treatments of heat-shocking. Another study, conducted by Peng, et.al., described the observation of spinal cord regeneration in zebrafish, where, after anesthetizing the fish in a solution of MS-222, an incision was made to access and cut the spinal cord.(38)

Our proposed experiment will consist of an incision in the tissue, and in contrast to the studies above, no tissue will be removed nor will the incision be deep enough to expose the spinal cord during the process of injury. The implant will be inserted completely subcutaneously within the intramuscular space. This is to allow a more rapid recovery and to decrease the wound size, reducing the amount of pain that will be experienced. Due to this parameter, our experiment is expected to inflict less pain than that of the Richardson, et. al. and the Peng, et.al. studies, therefore the treatments we have listed will be suitable for the assurance of complete anesthetization and pain management.

There have also been studies conducted that explore the use of post-operational analgesics, including the addition of isoflurane or lidocaine hydrochloride to the MS-222 solution to decrease potential side effects of MS-222 and to increase recovery time.(39,40) For this proposed experiment, the main focus is the examination of the natural immune reaction to the implant, therefore an induced recovery time may not provide accurate results of the neutrophil migration. Furthermore, in a microbead implantation procedure detailed by Gerlach et. al., as well as in a separate elastomer tagging procedure by Hohn and Petrie-Lanson, no additional analgesics were applied to the zebrafish after development into the juvenile and adult stages later in the experiments.(16,37) Therefore, it has been concluded that the proposed experiment does not have a bona fide alternative method through which to proceed, and that the methods proposed here are of comparable or refined pain management.

1. Arizona Game and Fish Department. Fish Tagging and Marking Techniques [Internet]. 2013 [cited 2017 May 9]. Available from: http://www.azgfd.gov/w_c/Fish_Tagging_Marking_Techniques.shtml/

2. Sciences B. PIT Tagging : Simple Technology at Its Best. 2017;54(5):447–54.

3. Baras E, Malbrouck C, Houbart M, Kestemont P, Melard C. The effect of PIT tags on growth and physiology of age-0 cultured Eurasian perch Perca flu Õ iatilis of variable size. 2000;

4. Rompen E. The effect of material characteristics , of surface topography and of

implant components and connections on soft tissue integration : a literature review. 2006;17:55–67.

5. Bo O, Pihlajama H. Clinical biocompatibility of biodegradable orthopaedic implants for internal " xation : a review. 2000;21.

6. Ambrose CG, Clanton TO. Bioabsorbable Implants : Review of Clinical Experience in Orthopedic Surgery. Ann Biomed Eng. 2006;32(1):171–7.

7. Jorfi M, Foster EJ. Recent advances in nanocellulose for biomedical applications. J Appl Polym Sci. 2015;132(14).

8. Morán JI, Alvarez VA, Cyras VP, Vázquez A. Extraction of cellulose and preparation of nanocellulose from sisal fibers. Cellulose. 2008;15(1):149–59.

9. Lin N, Dufresne A. Nanocellulose in biomedicine: Current status and future prospect. Eur Polym J [Internet]. 2014;59:302–25. Available from: http://www.sciencedirect.com/science/article/pii/S0014305714002493

10. Torres FG, Troncoso OP, Torres C, Grande CJ. Advances in Natural Polymers. Adv Struct Mater [Internet]. 2013;18:21–54. Available from: http://www.scopus.com/inward/record.url?eid=2-s2.0-84874283870&partnerID=tZOtx3y1

11. Abraham E, Deepa B, Pothan LA, Jacob M, Thomas S, Cvelbar U, et al. Extraction of nanocellulose fibrils from lignocellulosic fibres: A novel approach. Carbohydr Polym [Internet]. 2011;86(4):1468–75. Available from: http://dx.doi.org/10.1016/j.carbpol.2011.06.034

12. Prakash P, Kasaby HE, Aggarwal RK, Humfrey S. Microincision bimanual phacoemulsification and Thinoptx implantation through a 1.70 mm incision. Eye (Lond). 2007;21(2):177–82.

13. Fine IH, Hoffman RS, Packer M. Optimizing refractive lens exchange with bimanual microincision phacoemulsification. J Cataract Refract Surg. 2004;30(3):550–4.

14. Ahoor M, Sorkhabi R. Intraocular Foreign Body Removal by 23-Gauge Micro Incision Vitrectomy Surgery and Back Flush Flute Needle: A Case Series Study. Glob J Health Sci [Internet]. 2016;9(5):262. Available from: http://www.ccsenet.org/journal/index.php/gjhs/article/view/56079

15. Sambasivarao S V. OCT-Documented incision features and natural history of clear corneal incisions used for bimanual microincision cataract surgery. Cornea. 2013;18(9):1199–216.

16. Gerlach GF, Morales EE, Wingert RA. Microbead Implantation in the Zebrafish Embryo. J Vis Exp [Internet]. 2015;(101):e52943. Available from:

http://www.jove.com/video/52943/microbead-implantation-in-the-zebrafish-embryo

17. Brittijn SA, Duivesteijn SJ, Belmamoune M, Bertens LFM, Bitter W, De Bruijn JD, et al. Zebrafish development and regeneration: New tools for biomedical research. Int J Dev Biol. 2009;53(5–6):835–50.

18. Richardson R, Slanchev K, Kraus C, Knyphausen P, Eming S, Hammerschmidt M. Adult zebrafish as a model system for cutaneous wound-healing research. J Invest Dermatol [Internet]. 2013;133(6):1655–65. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23325040%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3644348

19. Chang W, Hwang P. Development of Zebrafish Epidermis. 2011;214(Part C):205–14.

20. Sullivan C, Kim CH. Zebrafish as a model for infectious disease and immune function. Fish Shellfish Immunol. 2008;25(4):341–50.

21. Lambris JD. Current Topics in Innate Immunity II.

22. Mathias JR, Perrin BJ, Liu T, Kanki J, Look AT, Huttenlocher A. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish flammation is a critical process during normal im-. 2006;80(December).

23. Rosales C, Demaurex N, Lowell CA, Uribe-querol E. Neutrophils : Their Role in Innate and Adaptive Immunity. 2016;2016:2–4.

24. Developmental stage data for this paper were retrieved from the Zebrafish Information Network (ZFIN); University of Oregon; Eugene; OR 97403-5274; URL: http://zfin.org/; 9/20/17.

25. Renshaw S, Loynes C. A transgenic zebrafish model of neutrophilic inflammation. Blood… [Internet]. 2006;108(13):3976–8. Available from: http://bloodjournal.hematologylibrary.org/content/108/13/3976.short

26. Lawson ND, Weinstein BM. In Vivo Imaging of Embryonic Vascular Development Using Transgenic Zebrafish. Dev Biol [Internet]. 2002;248(2):307–18. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0012160602907116

27. White RM, Sessa A, Burke C, Bowman T, Leblanc J, Ceol C, et al. Resource Transparent Adult Zebrafish as a Tool for In Vivo Transplantation Analysis. 2008;(February):183–9.

28. Karlsson J, von Hofsten J, Olsson P-E. Generating Transparent Zebrafish: A Refined Method to Improve Detection of Gene Expression During Embryonic Development. Mar Biotechnol [Internet]. 2001;3(6):0522–7. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/14961324%5Cnhttp://link.springer.com/10.1007/s1012601-0053-4

29. Tobias CW, Eisenberg M. IN ELECTROLYSIS-- REVIEW. 99(12):359–65.

30. Moore SW, Kennedy TE. Dissection and culture of embryonic spinal commissural neurons. Curr Protoc Neurosci. 2008;(SUPPL. 43).

31. Conrad GW, Bee JA, Roche SM, Teillet MA. Fabrication of microscalpels by electrolysis of tungsten wire in a meniscus. J Neurosci Methods. 1993;50(1):123–7.

32. Fang P, Lin JF, Pan HC, Shen YQ, Schachner M. A Surgery Protocol for Adult Zebrafish Spinal Cord Injury. J Genet Genomics [Internet]. 2012;39(9):481–7. Available from: http://dx.doi.org/10.1016/j.jgg.2012.07.010

33. OLYMPUS. Research Inverted System Microscope Ix71/Ix81 Ix2 Series. In: Manual.

34. Wikki Stix One of a Kind Creatables [Internet]. Available from: https://www.wikkistix.com/

35. Hachicho N, Reithel S, Miltner A, Heipieper HJ, Küster E, Luckenbach T. Body mass parameters, lipid profiles and protein contents of zebrafish embryos and effects of 2,4-dinitrophenol exposure. PLoS One. 2015;10(8):1–19.

36. Avella MA, Place A, Du SJ, Williams E, Silvi S, Zohar Y, et al. Lactobacillus rhamnosus Accelerates Zebrafish Backbone Calcification and Gonadal Differentiation through Effects on the GnRH and IGF Systems. PLoS One. 2012;7(9).

37. Hohn C, Petrie-Hanson L. Evaluation of visible implant elastomer tags in zebrafish (Danio rerio). Biol Open [Internet]. 2013;2(12):1397–401. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3863425&tool=pmcentrez&rendertype=abstract

38. Peng S, Yao L, Cui C, Zhao H, Liu C, Li Y, et al. Semaphorin4D promotes axon regrowth and swimming ability during recovery following zebrafish spinal cord injury. Neuroscience [Internet]. 2017;351:36–46. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0306452217301963

39. Collymore C, Tolwani A, Lieggi C, Rasmussen S. Efficacy and safety of 5 anesthetics in adult zebrafish (Danio rerio). J Am Assoc Lab Anim Sci. 2014;53(2):198–203.

40. Huang W-C, Hsieh Y-S, Chen I-H, Wang C-H, Chang H-W, Yang C-C, et al. Combined Use of MS-222 (Tricaine) and Isoflurane Extends Anesthesia Time and Minimizes Cardiac Rhythm Side Effects in Adult Zebrafish. Zebrafish [Internet].

2010;7(3):297–304. Available from:
http://www.liebertonline.com/doi/abs/10.1089/zeb.2010.0653

ASSURANCES FOR THE HUMANE CARE AND USE OF ANIMALS

As the Principal Investigator on this protocol, I assure that…

1) I have provided an accurate description of the animal care and use protocol to be followed in the proposed project/course.

2) the activities proposed do not unnecessarily duplicate previous experiments.

3) all individuals named in this application who are at risk will be registered in the Occupational Health and Safety Program.

4) all individuals performing animal procedures described in this application are technically competent and have been (or will be) properly trained in the procedures to ensure that no unnecessary pain or distress will be caused as a result of the procedures.

5) I will obtain approval from the IACUC before initiating any changes to this protocol.

6) I am familiar with and will comply with the *University of Maine's Policies and Procedures for the Humane Care and Use of Animals*, and I assume responsibility for compliance by all personnel involved with this protocol.

7) I have read and will follow the appropriate guidelines for the proposed species.

8) if using laboratory animals, all personnel handling the animals have had a tetanus shot within the past ten years.

9) all applicable rules and regulations regarding radiation protection, biosafety, recombinant issues, hazardous chemicals, etc., have been addressed in the preparation of this application and the appropriate reviews have been initiated.

10) animals will be purchased only from licensed, reputable vendors. If animals are purchased form a pet store, the pet store has been informed (in writing) that the animals will be used for research or teaching purposes.

11) I will maintain appropriate animal records (e.g., census, health, veterinary care, euthanasia, surgery, diagnostic, anesthesia, etc.)

12) **I will report at once to the IACUC any unanticipated harm to animals.**

13) I acknowledge that in the event of a disaster (natural or man-made) it may become necessary to triage, euthanize or otherwise modify the care and disposition of the study animals in order to avoid unacceptable pain or distress. I delegate overriding authority for emergency decisions of animal disposition to the Institutional Veterinarian or his/her designated representative.

Submission of the protocol indicates you have read and agree to the above Assurances

REMINDER: The Principal Investigator (PI) MUST submit the protocol. Another faculty member (no students) may submit the protocol on behalf of the PI with documentation of an email exchange that the PI has read and approves. We require this because the PI is ultimately responsible for the content of the protocol submission.

AUTHOR'S BIOGRAPHY

Hanna Joy Anderson was born in Dover-Foxcroft, Maine on August 17, 1995. She grew up in Brewer, Maine and graduated from Brewer High School in 2013. Along with her Bioengineering major, Hanna is also minoring in Nanotechnology. She is a member of Engineers Without Borders, University of Maine Chapter, and served as the secretary of the University of Maine Bioengineering Club. She received a United Studies Abroad Consortium scholarship and studied abroad in Alicante, Spain during the summer of 2014. Hanna plans to further her education for an advanced degree in Biomedical Engineering, and is currently applying to graduate schools around the United States.