ABSTRACT

Title of Document:	PHYSICAL PROPERTIES OF LAMPREY SPINAL CORD REGENERATION: ADAPTIVE VS. MALADAPTIVE RECOVERY
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Spinal cord injury (SCI) is a physical trauma that can result in paralysis and even death; to date no treatment exists that can successfully promote functional or adaptive recovery. Although humans are unable to regenerate after complete SCI, there are animal models that have been studied for their ability to regrow and reconnect their nerve fibers.

From the group of animals that are capable of spinal cord regeneration, in the best studied is the lamprey *(Petromyzon Marinus)* it has been noted that recovery can be maladaptive. When left to recover at warm temperature (23 °C) most lampreys had adaptive behavior, but at cold temperature (10 °C) most lampreys showed maladaptive behavior. In this dissertation we studied the physical factors that influence adaptive and maladaptive recovery in lampreys.

In the first part, we analyzed axonal regeneration and blood clot formation at early time points after injury (1-2 weeks). We found that lampreys in cold temperature have a blood clot that could be blocking spinal cord regeneration.

In the second part of this work, we analyzed the biomechanical and structural differences between lampreys in warm and cold temperature. We used in vivo X-ray imaging and tensile loading testing of the spinal cord and notochord structures, before and after injury. We found that lampreys at warm temperature are more favorable to create a permissive mechanical and structural environment for regeneration.

Lastly, we used those lessons learned previously to enhance regeneration of maladaptive animals. We removed the blood clot at the injury site and created a time frequency analysis to measure the recovery of coordination. We found that lampreys in cold temperature with clot removal had a more adaptive recovery after injury than those without removal.

In summary, by using the lamprey we were able to compare the differences between regeneration in warm and cold temperature and found the physical factors that influence maladaptive recovery. Removing one of these factors, in this case the blood clot, successfully enhanced the recovery of coordination. These results have the potential to be translated to higher animals and aid in the creation of successful treatments for SCI.

PHYSICAL PROPERTIES FO LAMPREY SPINAL CORD REGENERATION: ADAPTIVE VS. MALADAPTIVE RECOVERY

By

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Preface

Injuries to the spinal cord are some of the most devastating traumas to the human body. The spinal cord controls motor and sensory functions in the body and it is the main connection between the peripheral nervous system and the brain, complete transection of the spinal cord can cause paralysis and even death.

Although the ideal model to study would be humans, injuries to the spinal cord are extremely complex and involve a great number of tissues. Furthermore, the scarring process leaves an almost unrecognizable blockage at the injury site, from which there are many cellular and molecular factors virtually impossible to study independently.

These facts have made researchers look for animal models with more controlled variables. It was observed that some animals had the innate capability to regenerate the spinal cord and in this group we can find animals such as zebrafish, goldfish, newt and lamprey.

Danish physiologist August Krogh stated that for such a large number of problems there will be some animal of choice, or a few such animals, on which it can be most conveniently studied. In our case we decided to use the lamprey because we discovered that its recovery of locomotion wasn't perfect, but more importantly, that we could control the degree of recovery. When placed in warm temperature (23 °C) recovery was mostly adaptive and in cold temperature (10 °C) was mostly maladaptive. This gave us the opportunity to analyze what are the physical factors

that tip the balance on lampreys and makes them either adaptive or maladaptive. We had the idea that if we could understand their differences, we could study how to tip the balance ourselves by engineering a treatment. Furthermore, understanding how lampreys become maladaptive could give us some clues on how to avoid this outcome in other animal models.

Therefore, this thesis was created with the purpose of understanding the differences between adaptive and maladaptive regeneration. In the second chapter we introduce the structural properties of adaptive and maladaptive regeneration, where we discovered that a blood clot blocks regeneration in cold, but not in warm temperature. In the third chapter we explored the mechanical and structural properties of the spinal cord and the notochord. We found that only animals in warm temperature have similar mechanical properties to that of control, indicating that at this temperature the tissue forms a permissive environment for regeneration. Lastly, we used the lamprey as a way to test head to tail coordination after removing the blood clot in cold temperature. We found that removing the blood clot increased the coordination between head and tail when compared to animals without removal. The lamprey has been a great, simple model in which we were able to study and change the properties that tip the balance between adaptive and maladaptive recovery.

All of the work presented henceforth contains the result of research undertaken at the Cell Biophysics Laboratory, University of Maryland. All projects and associated methods were approved by the Institutional Animal Care and Use Committee at the University of Maryland (Protocol R-10-97).

Dedication

To my parents, Carlos Luna and Silvia Lopez

Acknowledgements

I would like to thank my advisor Dr. Helim Aranda-Espinoza for the opportunity to work at the Cell Biophysics Laboratory, his important feedback helped me better understand the importance of biomechanics and cell mechanics.

Special thanks to my co-advisor and mentor Dr. Avis. H. Cohen who introduced me to the field of Neuroscience and whom without any of the lamprey work wouldn't have been possible. Her advice has been key for my development as a scientist and as a person.

I am thankful to Dr. Sameer Shah, who taught me the procedure to measure the mechanical properties of the spinal cord and discussed with me the results which lead to a publication.

Many thanks to the committee members Dr. John F. Fisher, Dr. Adam H. Hsieh and Dr. Sergei Sukharev for their important feedback throughout my Ph.D. work.

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Specific Aims

Main hypothesis

We hypothesized that the mechanical and structural properties of the spinal cord are different between animals with adaptive and maladaptive recovery. Our goal was to understand these properties and used those lessons learned to enhance recovery of animals to a more adaptive endpoint.

Aim: 1. Adaptive vs. Maladaptive recovery after spinal cord injury

We characterized, spatially and temporally, the spinal cord lesion early after injury using immunostaining and histology. We hypothesized that the functionality of regeneration is determined by structural changes early (2 weeks) after injury. We discovered that the main difference between adaptive and maladaptive animals was the presence of a blood clot at the injury site that might hinder regeneration in cold temperature.

Aim: 2. Mechanical properties of the spinal cord and notochord after injury

We hypothesized that the mechanical environment in adaptive animals was permissive for regeneration, but not in maladaptive. We found that the spinal cord was under a pre-strain condition that induces recoil after injury. The mechanical properties of adaptive animals were more similar to uninjured animals. The notochord was unable to recover normal mechanical and structural properties in maladaptive animals.

Aim: 3. Blood clot removal on animals in cold temperature

We hypothesized that removing the blood clot would enhance recover of animals in cold temperature (maladaptive). Using a time frequency analysis we found that the blood clot removal effectively enhanced regeneration to a more adaptive endpoint for animals regenerating in cold temperature.

Chapter 1: Spinal cord injury, animal models and lamprey spinal cord regeneration

1.1 Human Spinal Cord Injuries

The nervous system of humans and most vertebrates is composed of a central nervous system (CNS) formed by the brain and the spinal cord which control most of our movements and organs. Extending from the spinal cord is the peripheral nervous system (PNS) which forms the necessary connections for the communication between the CNS and the rest of the body.

The central nervous system is protected by a vertebrate column (spinal cord) and cranium (brain); below these bony structures, the CNS is protected by a set of membranes (the meninges). These structures are formed by a complex set of cellular components, named glia; which include astrocytes, oligondreocytes, ependymal cells and microglia. In order to support nutrition, an even more complex set of blood vessels run parallel and into the spinal cord in small branches. In contrast, the PNS doesn't have a protective membrane or bone layer, leaving it open to reach muscles and organs [1]. Through evolution, our body has developed the ability to heal and regenerate in response to injuries and diseases. For example, inflammation, tissue formation and remodeling [2] lead to a rapid regeneration after most cutaneous injuries. However, it

is the fascinating complexity of the CNS that also contributes to its poor regenerative capabilities.

According to the National Spinal Cord Injury Statistical Center (NSCISC) there are 250,000 Americans with spinal cord injury. Most of these injuries occur in young males between the ages of 16 and 30, meaning that they have most of their life ahead. The cost of managing the care of spinal cord injury patients approaches $4 billion dollars a year.

These injuries are caused by everyday life actions, such as driving motor vehicles (48%), falls (21%) and sports (14%). They can be categorized into complete and incomplete. Complete injuries result in total loss of sensation and function below the injury level and incomplete result in partial loss of function. Furthermore, patients with complete lesions above C-3 vertebrae die before receiving medical treatment and those who survive depend on mechanical respirators to breathe. The classification of each injury, its symptoms and outcomes are determined by the American Spinal Injury Association (ASIA) [3]. The need for such an extended classification is correlated to the complexity of SCI which is one of the most unforgiving traumas to the human body. In order to establish the rationale for our research and to set up the structure of our aims, we will explore the temporal and structural stages of SCI.

1.1.1 Causes and consequences of spinal cord injuries

Catastrophic falls, such as horse-riding accidents; violent encounters (knife, bullets), car accidents and/or sports injuries cause bones forming the vertebrae to be broken or dislocated causing a traumatic injury to the spinal cord (Figure 1-1). Most injuries to the spinal cord are compressive, causing the axons to be crushed by the vertebrae (Figure 1-1b), while others completely sever it (Figure 1a). Since the spinal cord is the main pathway for information, depending on the level of injury, SCI patients can be quadriplegic (paralysis of most of the body) or paraplegic (paralysis of the lower trunk and legs).

Figure 1-1. Magnetic resonance imaging of spinal cord injuries in human patients. a) Cervical dislocation with complete transection of the cord. b) Compression of spinal cord due to disc hernia, image modified from [4].

1.1.2 Post-injury episodes

The damage to the spinal cord after a traumatic blow, bruises or tears spinal cord tissue, breaking apart axons and bodies inflicting a catastrophic neuronal damage.

This just indicates the beginning of the damage; this physical trauma sets off a cascade of events that continue for days and will influence the patient's outcome and future treatments.

Homeostasis and blood pressure. Within minutes, spinal cord swelling cuts \off blood flow, which cuts oxygen and blood pressure to the point the body can't self-regulate (hypothermia [5]) and interferes with neuronal activity, causing a *spinal shock* which can last from hours to days [6]. During spinal shock, even undamaged portions of the spinal cord are temporally disabled, making it more difficult for the doctor to establish a diagnosis of the trauma and the hypothermia events will last during the normal life of the patient [5].

Neurotransmitters. After the injury, an excessive release of neurotransmitters causes additional damage by overexciting cells, causing spasms [7] with can lead to secondary injuries and even death. However, neurotransmitters are necessary for successful recovery and future treatments include the integration of neurotransmitters into biodegradable scaffolds [8].

Apoptosis. Death of cells is not limited to the initial trauma, as programmed cell death (apoptosis) can occur weeks after the injury [9]. SCI leads to increased expression of tumor necrosis factor and its receptor due to inflammation which affects cell death; however, there are conflicting results as to the role of cell death after SCI [10].

Immune response. After SCI, the blood brain barrier no longer controls the passage of cells and large molecules, leaving a free pathway for immune cells to the injured tissue, triggering an inflammatory response. This inflammation is characterized by the influx of immune cells, including neutrophils, T-cells, macrophages, and monocytes *[11]*. The beneficial effect of this response includes fighting infections, controlling immune response and cleaning up debris. Detrimental consequences involve the expression of inflammatory cytokines by microglial cells [12] and stimulate astrocytes which ultimately participate in the formation of scar tissue [11]. Cellular and molecular components of scar tissue are one of the most studied subjects in SCI [13], with conflicting results on the beneficial [14] and negative effects [15].

1.1.3 Consequences and secondary damage

The destruction of axons and inflammation response sets the beginning of the devastation of SCI, which continue for weeks and years after the injury. Secondary damage can exacerbate the injured area and also the extent of disability. In fact, most SCI are detected or treated weeks after the initial trauma, when the inflammation process and scar formation is already advanced [14]. At this point, glial cells have invaded the injury site forming a scar, which creates a barrier for axonal outgrowth [15]; even if few axons remain, in most cases there is not enough to send any meaningful information to the brain. Researchers are especially interested in studying the mechanisms of this wave of secondary damage because finding ways to stop it could save axons and reduce disabilities. This could make a big difference in the potential for recovery.

1.1.4 Treatment options and future therapies

SCI is commonly related to paralysis; however medical complications after SCI [16] include chronic pain, respiratory and heart problems, spasms [7], blood clots, low blood pressure and hypothermia [5]. These problems represent not only the lack of connectivity from injured axons, but also the involvement of the many other cellular mechanisms along the spinal cord, which play an important role in successful recovery.

The most used treatment options for SCI are limited to control of secondary symptoms: relief of compression on the spinal column [17], temperature treatments [5] and prevention of secondary injuries [18] with anti-inflammatory drugs [19] such as methylprednisolone [20]. Other treatments such as antibody blocking of inhibitor molecules [21, 22] offer a new hope for SCI patients; however there is still much to know about their primary and secondary effects. Enzyme treatments against inhibitory molecules (chondroitin sulfate proteoglycans (CSPG)), such as chondroitinase ABC [23] have been show to promote axonal outgrowth for the peripheral nervous system and are a promising treatment for the CNS.

The increasing knowledge on each of the cellular mechanisms that affect the spinal cord gives scientist better options to treat SCI. Medical treatments such as decompression, neural prostheses [24] and scaffolds [25] involve an invasive surgery weeks after SCI, in which the scar tissue might be partially or completely removed. Unwillingly, the treatment represents a secondary injury to the already damaged tissue;

as for the collateral effects of this procedure, nothing is understood. In fact, it has been shown that peripheral neurons are 100 times more likely to regenerate into peripheral grafts if they have previously been through a "conditioning" injury [26]. This ability has been related to the up-regulation of regeneration-associated genes [27] and improvement of sprouting [28]. The closest surgical scenario of is the treatment to relieve back surgery pain (laminotomy), which removes tissue, formed by back surgery or herniated disks [17]. This procedure includes the risk of neuronal damage and increased formation of scar tissue.

1.1.5 Current clues to the unsuccessful regeneration of the CNS (Glial Scar)

Why are humans unable to recover from SCI? Spinal cord regeneration is not a problem specific to the neurons; extensive research on CNS injury has provided clues of factors that are involved during injury [13-15]. The most critical of all is the formation of a glial scar, which in humans is seen as a "barrier" for regeneration. Reactive gliosis (scar formation), is a process that repairs the wound, but it forms a tissue that is not permissive for axonal regeneration as compared to normal tissue from the spinal cord which promotes axonal outgrowth [29]. The inhibitory property of scar tissue can come from its physical properties such as stiffness to the expression of attractant and repellant molecules. The most important of them are myelin-associated proteins (e.g. netrins) and proteoglycans (CSPGs) which are present in the scar.

The extracellular matrix (ECM) of spinal cord and brain tissue during development has some of the common ECM proteins (fibronectin, laminin, and

collagen); however at later stages these molecules are scarce and restricted to the outside parts of the cord (blood vessels and meninges) [30, 31]. Thus, it is believed that the components of the CNS matrix are molecules such as proteoglycans and other proteins expressed by oligodreocytes (called myelin-associated) [32].

Chondroitin sulfate proteoglycans are one of the major classes of proteoglycan present in the CNS expressed mostly after the formation of the scar by reactive astrocytes [33]. They consist of a protein core to which many glycosamingylcan (GAG) chains are covalently attached. These chains give CSPGs most of their properties [33] and are useful for treatment [23] and general localization with antibodies. CSPGs were believed to be inhibitory molecules of axonal growth, found in regions where growth cones are absent. However their function has shown to be much more complex and the concept of CSPG "inhibition" has been changed to "guidance". For example, depletion of CSPGs in cultured retina results in lack of directionality from extending axons [34].

In summary, lecticans, aggrecan [35, 36], versican [37] neurocan [38] and bervican [39] CSPGs have been linked to inhibitory functions due to the GAG chains, whereas NG2 is inhibitory. Phosphacan has been shown to promote growth in cortical neurons but not in thalamic neurons and DSD-1-PG promotes growth of mesencephalic and hippocampal neurons [40]. All these studies show the dual role of CSPGs and how it might depend on their localization.

Similar to the molecules expressed by reactive astrocytes, myelin-associated proteins are molecules expressed by oligodendrocytes that are related to the inhibition of axonal regeneration. Inhibitor molecules Nogo [41], myelin-associated glycoprotein (MAG) [42] and oligondreocyte myelin glycoprotein (OMgp) [43] have been found to be associated with inhibition, and recent data has associated these proteins with a common receptor (Nogo receptor, Ngr). Several treatments have evolved from these discoveries; however, immunizing against myelin proteins might have degenerative properties overall. Netrin-1 is another myelin-associated protein that can either attract or repel axons depending on the combination of receptors expressed in neurons (UNC5 and DCC receptors) [44]. More importantly, these receptors have been found in the lamprey [45], and thus will be the objective of our first studies on myelin-associated proteins.

1.2 Animal Models for SCI

In mammalian models, great technical effort must be expended to achieve none or a relatively small amount of regeneration and to gain both regrowth and functional improvement. “Lower” vertebrates on the other hand, are known to show axon outgrowth after injury [46]. This axonal outgrowth is usually stated as regeneration; however, we must be careful with this statement. Reading through several reports, we have found that not surprisingly, these assumptions are commonly incorrect and/or exaggerated; therefore, it is important to differentiate between axonal outgrowth and regeneration. Functional regeneration is achieved when all elements of the spinal cord act to recover normal behavior; this includes not only neurons but also glial cells and CNS molecules involved. In the following paragraphs, we will analyze the rationale for our animal model, first looking at the goldfish and zebrafish models.

Some lower vertebrates used to study CNS regeneration include the goldfish [47-49], zebrafish [50-52], eel [53, 54] and newt [55, 56]. The goldfish (*Carrasius auratus*) is one of the most studied animals for neural regeneration. Variability has been found on the growth of fibers; sometimes only 50% of the fibers can regenerate after axotomy and only 60% of the propiospinal neurons are involved in recovery [57]. Furthermore, the degree of functional recovery is limited and often leads to incorrect pathways [58]. The next best model is the zebrafish, an animal model with a lot of genetic tools, ideal for studying vertebrae development [59]. The suitability for molecular studies has caught significant attention for the use of zebrafish to study CNS

regeneration [60]. Goldfish and zebra fish have a big disadvantage: their locomotor system has not been fully described, which makes it difficult to establish when adaptive recovery has been achieved. In this thesis we are interested not only in the regrowth of the spinal cord, but in the different behavioral outcomes after injury.

Regeneration is imperfect, it is incomplete. In other words, using an animal model for regeneration does not mean that the spinal cord will regenerate exactly as it was before injury. In the goldfish, only 30% of fibers regenerated and were functional [57]. However, many of these regenerated axons changed their original target in the CNS to the peripheral nervous system (PNS), which left many goldfish unable to recover normal behavior [58]. The eel [53] and the lamprey [61] have been shown to regenerate only a few fibers as well, around 20%. In the zebrafish, 32-51% of the neurons with descending axons were able to regenerate and only 2-4% neurons with ascending axons regrow [62]. Newts did not regenerate sensory axons and only few fibers from the spinal cord regenerated [56]. In the weakly electric teleost fish, only 20% of regenerated cells are neurons, the rest were found to be glial cells [63].

Recovery is imperfect, it is not typically adaptive but it can be maladaptive. In other words, animal models that are used for spinal cord regeneration do not always recover normal behavior after injury. Behavioral studies on the newt indicate that only 14% of 6-week and 67% of 9-week regenerated newts recovered adaptive behavior [56]. Other studies in regenerated salamanders showed that their locomotor patterns suffer transient and long term alterations after injury, more during swimming than

during walking [64]. Weakly electric fish that regenerated in 30 ºC vs 22 ºC showed different functionality. Those that regenerated in 30 ºC were able to create an electric discharge more than double the rate than those at 22 ºC [65]. Low levels of L1.1 protein impair locomotor behavior, reduce the rate of regeneration and the formation of synapses in the zebrafish [66]. Incomplete and maladaptive spinal cord regeneration are two obstacles that researchers must understand and master in animal models before achieving regeneration in humans. Although it is clear that these two obstacles are present in different animal models, it is often unheard of or undiscussed to talk about them. In this thesis, instead of a disadvantage, we will use the fact that lampreys have adaptive and maladaptive regeneration to our advantage by creating a set of comparative studies to better understand the factors that can change the outcome after spinal cord injury.

1.3 Lamprey and the recovery of locomotion

1.3.1 Lamprey anatomy and physiology

Lampreys (*Petromyzon marinus*) also known as petromyzontids are one of the few extant vertebrates that originated over 500 million years ago (Figure 1-2 b). Although they don't have a bony skeleton, they are true vertebrates placed in a basal position on the evolutionary tree (Figure 1-2 a), thus they are mostly generalized as basal or lower vertebrates. They are eel-shaped vertebrates (Figure 1-3) that live in the sea but migrate to rivers to spawn and lay eggs [67]. Lampreys attach themselves to other fish by means of a set of horny teeth that surround the mouth (Figure 1-3).

They have a notochord (Figure 1-4) with an overlying spinal cord flanked with a pair of arcualia on each side. The spinal cord runs longitudinally across the animal and the dorsal and ventral roots are segmented along the length of the animal [67]. At the anterior end of the spinal cord lies the brain, which is elongated in shape (Figure 1-3). The brain has the main structures of primitive brains, a cerebrum, cerebellum, optic lobes, rhombencephalon and pineal organs that are photosensory (Figure 1-3) [67].

Lampreys have been used as animal models for the study of motor control [68-70] and due to their ancestry, as animal models for the study of evolution [71]. It was until recently that the regenerative capabilities of the lamprey have been explored. More importantly, it was until recent experiments involving nerve recordings during fictive swimming; that the functionality of lampreys was measured and it was discovered that their recovery was not always adaptive [72]. Thus, leaving us to question what factors could be different in those animals that didn't recover adaptive behavior.

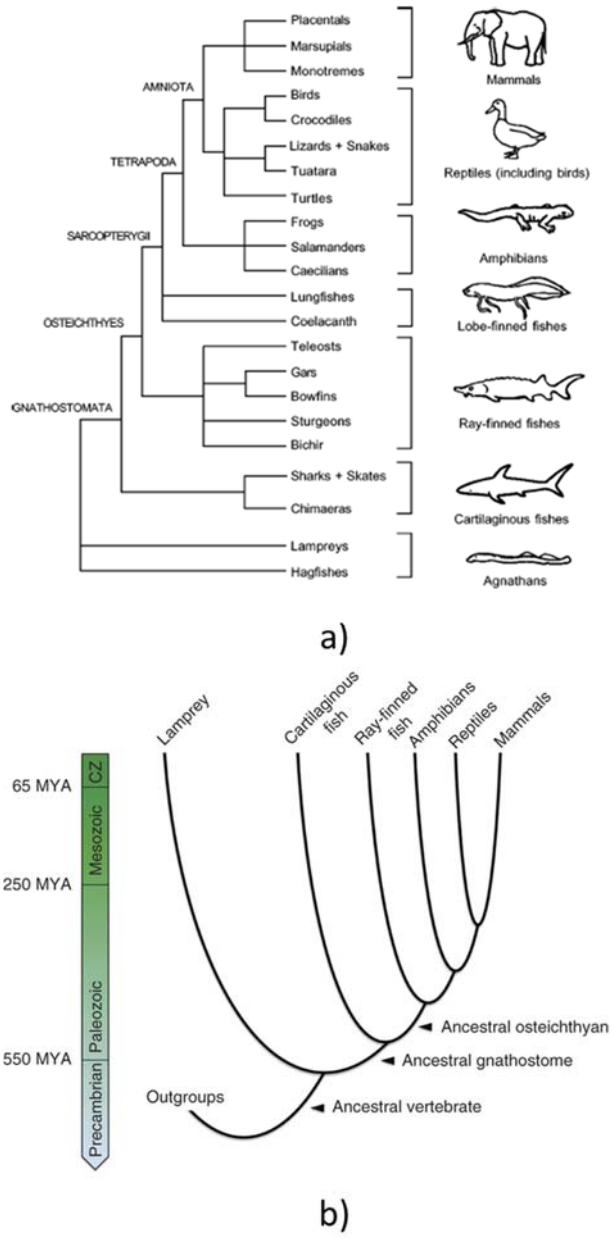


Figure 1-2. Phylogenetic tree of lampreys. a) Tree for the major lineages of vertebrae based on morphological and paleontological evidence, image modified from [73]. b) Timing of major events within the vertebrae lineage. CZ, Cenozoic; MYA, million years ago, image modified from [71].

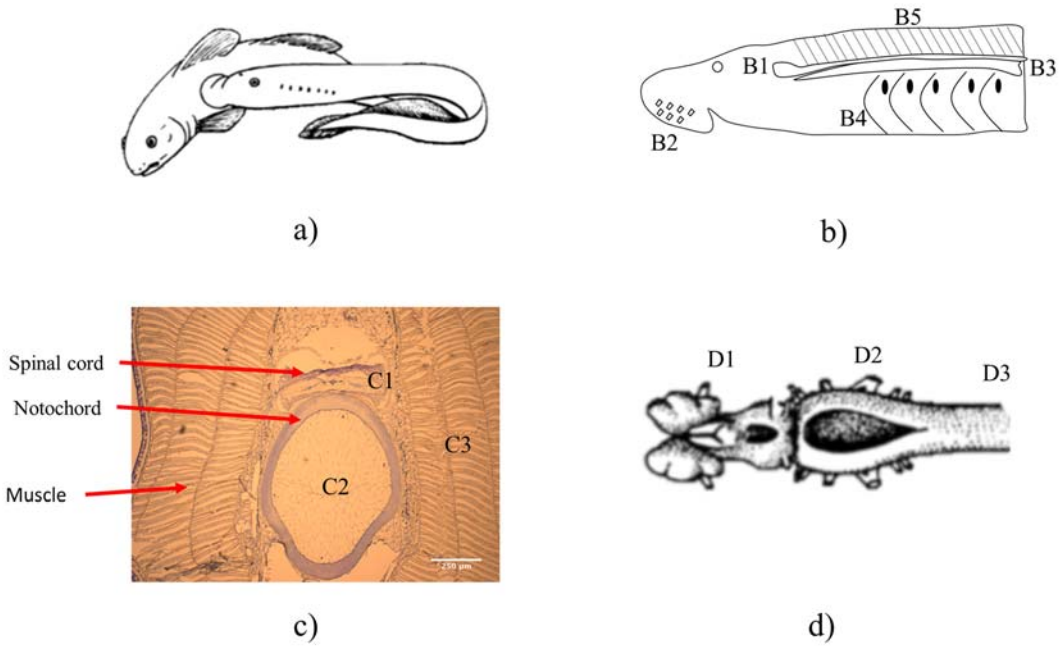


Figure 1-3. Characteristics of the lamprey. a) Adult lamprey feed by attaching themselves to fish extracting their blood, b) Lampreys are jawless vertebrates with a set of filters in their mouth for feeding (B2). They have a brain and spinal cord (B1) that sits on top of a notochord (B3), the notochord is surrounded by muscle (B5). Their breathing is through a set of gills located at the head (B4). c) Cross section of the lamprey stained with toluidine blue, scale bar is 250 µm. (C1) Spinal cord, (C2) notochord that serves as the axial support with the surrounding muscle (C3). d) The lamprey brain consists of a set of olfactory bulbs and optical nerves and a cerebellum at the upper part (D1), then there is the medulla oblongata and the spinal nerves (D2) which connect to the spinal cord (D3). Image modified from [67].

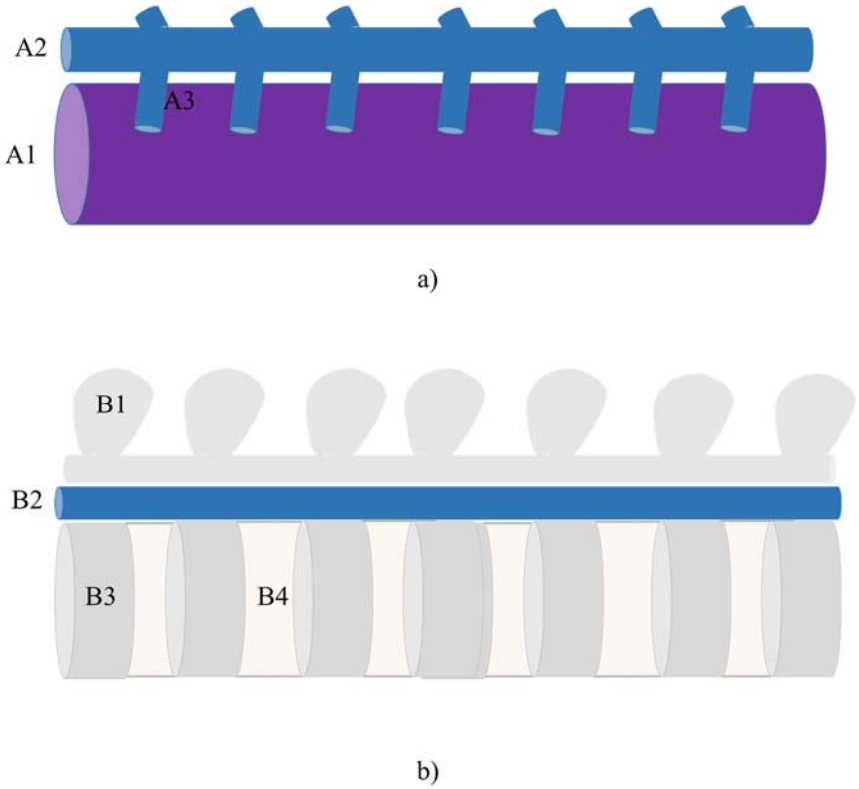


Figure 1-4. Hydraulic skeleton vs. Bony skeleton. a) Lamprey have a hydraulic skeleton that consists of a notochord (A1) which consists of a set of vacuolated notochord cells enclosed by a collagen sheath, in top of the notochord sits the spinal cord (A2) which extends a set of dorsal and ventral roots per segment (A3). b) Humans have a bony skeleton which consists of a set of calcified vertebrae (B3), intervertebral discs (B4) and facet joints (B1). In the case of humans, the spinal cord (B2) can be found protected in between these structures, unlike the lamprey in which the spinal cord just lays on top the notochord. Image modified from [67].

1.3.2 Spinal cord regeneration

What makes the lamprey a good model? Lamprey is a model for spinal cord regeneration; it has been subject of many locomotion, electrophysiological and histological studies [74-83]. Their spinal cord is governed by the central pattern generator (CPG), a feature common to most vertebrates, including humans. This feature has gained the interest of many researchers to treat spinal cord injuries in which the connection between brain and spinal cord has been broken. By installing a controlling circuit in the spinal cord, with a similar pattern to that of the lamprey, the CPG of humans can react and thus recover locomotion [24]. There are many characteristics of lamprey regeneration that can be relevant to human spinal cord injury; we have summarized some in Table 1-1.

The same overstatement that regeneration is always perfect, might be occurring in the lamprey model as well. It was in fact not until recently that adaptive and maladaptive conditions were differentiated [72]. Locomotion studies have shown that lampreys recovering at cold native temperatures (10°C) tended to have a maladaptive behavior and levels of 5-HT were always below control uninjured animals (Figure 1-5 and 1-6). When the lamprey was left to recover at room temperature (23°C), the recovery was typically adaptive, locomotion and 5-HT levels were fully recovered as in control animals (Table 1-2) [84]. Perhaps, the properties of the injury site in the cold lampreys are more human-like and thus inhibit regeneration. However the differences between these two conditions haven't been explored and are part of the objective of this work.

Property	Humans	Lamprey
Regeneration time	Changes in myelin 10 days after injury and axonal swelling 4 weeks after [85]	Die back for 2 weeks [76] and functional regeneration after 25 weeks [77]
Degeneration process	Wallerian Degeneration [85]	Degeneration [86]
Neurofilaments	Yes	Yes [87]
Glial Cells	Reactive astrocytes, Oligondreocytes, Blood Cells, Microglia	Astrocytes (not known if reactive), Oligondreocytes, Blood Cells, Microglia [86]
Receptors	Receptors (UNC5, DCC) found in rat for human netrin [88]	Netrin receptors (UNC5, DCC) [45]
Myelin	Yes	No [89]
Neurotransmitters	Serotonin, GABA, dopamine	Serotonin, GABA, dopamine [90-92]
Control-Information	CPG and brain	CPG and brain
Pathway of regeneration	There is no regeneration in the CNS	Regenerate to make functional synaptic connections [93]
Guidance mechanisms	Glial scar proteins (e.g. CSPGs) [33]	Unknown, but they show directional sensitivy which suggests the prescence of guidance molecules [94]

Table 1-1. Similarities between human and lamprey spinal cord injury.

	Condition	Lesion	Degradation	Recovery	Serotonin Levels
First Injury	Adaptive (25°C)	Midbody	5 wks	10-25 wks	5-HT sprouting surpases control levels
	Maladaptive (10°C)	Midbody	10 wks	25 wks	5-HT never reaches control levels

Table 1-2. Lamprey spinal cord regeneration at different temperature, data from [56].

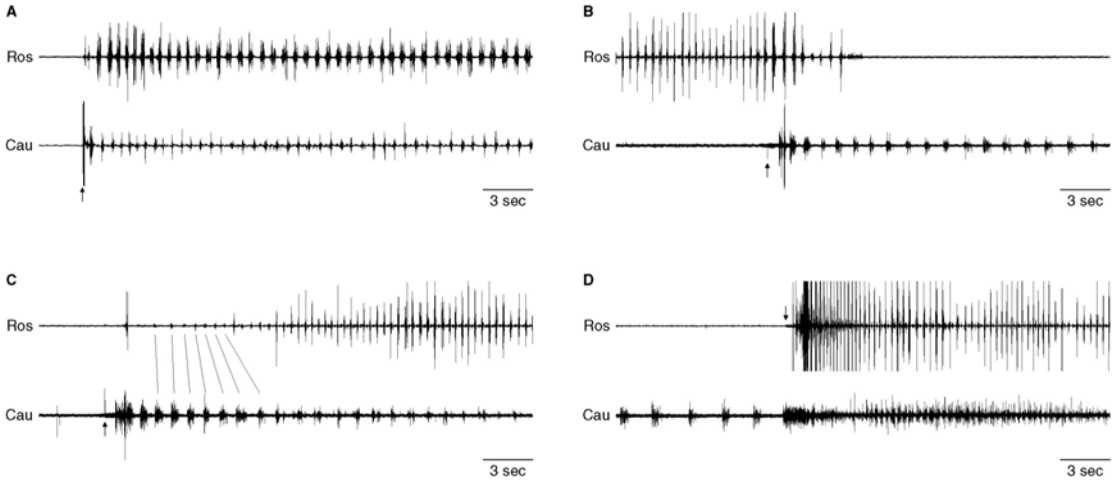


Figure 1-5. Electromyography of animals after spinal cord injury. Whole animal swimming in adaptive and maladaptive animals. a) Adaptive, b-d) Maladaptive. Recordings were done at the rostral and caudal parts after recovery from a spinal cord injury to the mid-body. Image modified from [56].

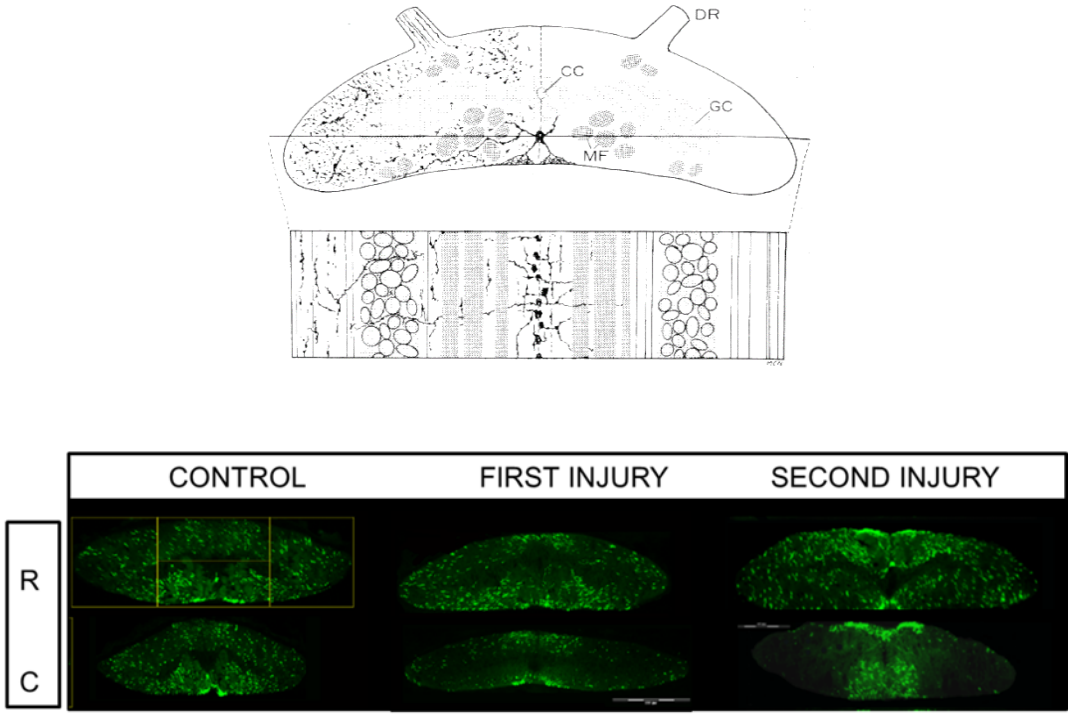


Figure 1-6. Serotonergic process and expression in the lamprey spinal cord. (Up) Figure of serotonergic processes in the intact lamprey spinal cord (transversal and whole mount sections, modified from [95]), CC=Central canal, DR=Dorsal roots, GC=Gray columns, MF= Giant Muller fibers. (Down) Expression of 5-HT on transversal sections of the spinal cord from animals recovered at cold temperature (maladaptive) for control, first and second injury. R=Rostral and C=Caudal (data from Dr. Cohen, unpublished).

1.4 Mechanical properties and structure of tissues involved in growth and regeneration

1.4.1 Mechanical properties and axonal outgrowth

An integral element on SCI research is the study of spinal cord mechanical properties [96]. The relationship between SCI, regeneration and biomechanics can be

convened in four concepts: i) the spinal cord fibers have a pre-stress state, ii) strain of nerve fibers can impact physiological function [97, 98], iii) mechanical response might depend on the fibers, matrix and meninges [99-101], iv) axon regeneration may be enhanced with the appropriate mechanical tension [102] and environment [103, 104].

The importance of tensile loading on nervous tissue starts during development, where neurons and axons undergo changes in tension during growth and network formation [105, 106]. Therefore, it is no surprise that fiber strain has such an important role in the study of spinal cord function [107], injury [16, 108, 109], and repair [96]. Human studies are limited to computer simulations [110-112] and experimental techniques are limited to cadavers, with variation of the modulus (0.5-1.5 MPa) [101, 113, 114]. Other animal models have been used, such as bovine (0.94-1.66 MPa).

1.4.2 Notochord: Scaffold and axial support

More than 500 million years ago, prehistoric animals initiated the first stage in evolution of an endoskeletal backbone with the formation of a notochord [67, 115]; a fluid filled collagen rod [116] that served as a hydraulic skeleton [117]. Today, all chordates [118] such as birds, reptiles, fishes, amphibians and mammals, including humans, share this evolutionary stepping stone at some point in their life cycle [119-121]. For some of these species, the notochord develops during the embryonic phase and becomes part of the vertebral column, a segmented, calcified backbone [120, 122-126].

Recent studies that have shed light on the origins of vertebrate elements [127-129] and the development of the axial skeleton [130, 131], demonstrate that our knowledge of the first stages of evolution [121] is far from complete. The notochord is a key structure to understand the evolution and development of the backbone (Figure 1-7), as evidence suggests it has an important role in the successful development of the vertebral column [121, 125], forming part of the nucleus pulposus [132] of intervertebral discs and is involved in the production of proteoglycans.

Early research on amphioxus notochords pointed to it being a specialized hydraulic skeleton with a muscular segmented nature [133]. However, similar specializations were not found in other extant chordates, such as the hagfish, in which notochord was shown to be a continuous structure [126]. In the zebrafish, the development of the spine starts with the mineralization of the notochord via calcified ring-shaped structures [134, 135]. This segmentation process of the notochord is only known to occur during the development of vertebrae and thus, it is not expected for basal vertebrates such as lampreys. In fact, it is generally accepted that the notochord is a continuous sheet of collagen fibers with no segmentation

The notochord has at least two known important roles [136]: 1) It serves as a scaffold for surrounding tissues by releasing chemical triggers during development that signal surrounding tissues such as blood vessels [137, 138] and cell types forming somites [139]. 2) It also serves a mechanical role by as the axial support during locomotion [136, 140-142]. Apart from the developmental stage, another process

which requires the use of a scaffold and a mechanical support is regeneration after spinal cord injury. We expect that the use of larvae lampreys will yield more clues on the role of the notochord in the process of regeneration, and help us understand the mechanical roles of a segmented sheath.

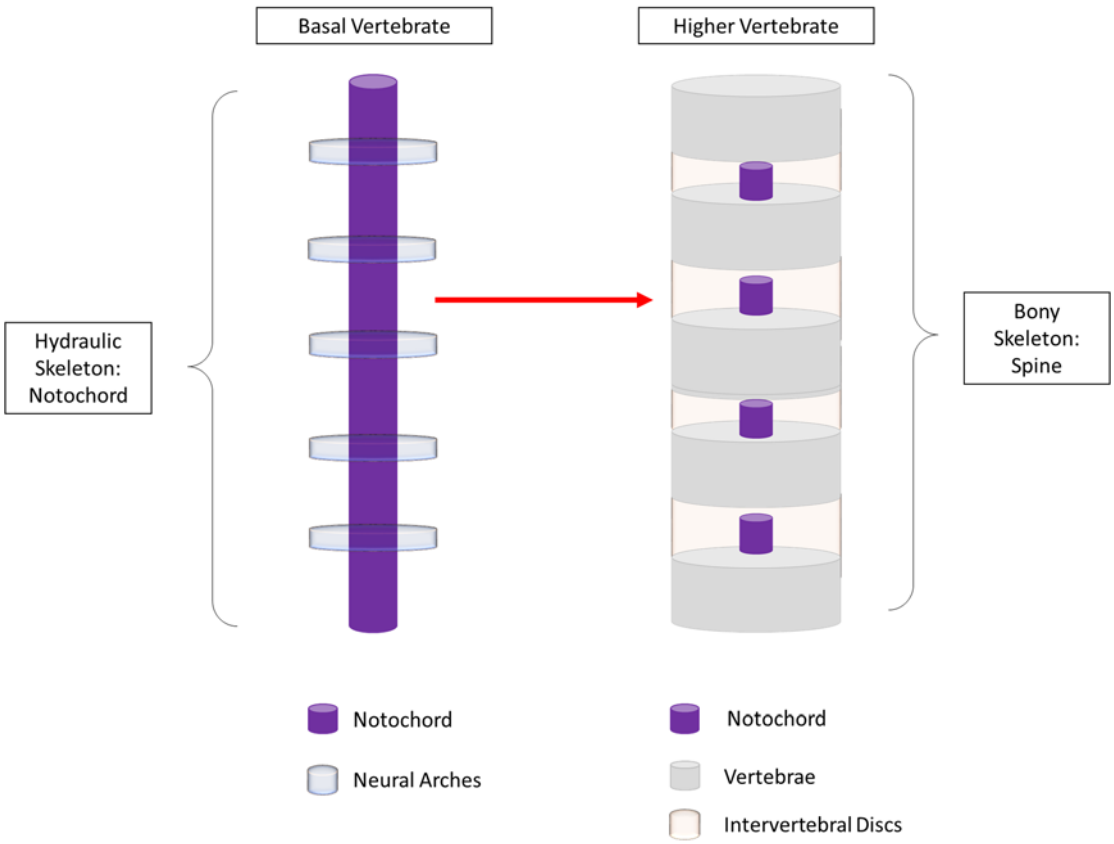


Figure 1-7. Development and evolution of the notochord. Basal vertebrates have a notochord that functions as their hydraulic skeleton and axial support. Higher vertebrates have a bony skeleton made of calcified vertebrae and intervertebral discs. Inside the intervertebral disc, in the nucleus pulposus we can find the remnants of the notochord that develops during embryonic stage.

Chapter 2: Axonal regeneration and clot dynamics of adaptive and maladaptive regeneration

2.1 Experimental Methods and Rationale

2.1.1 Rationale

Spinal cord injury (SCI) is a physical trauma that can result in paralysis and even death, with no treatment for humans to promote recovery. Lampreys can typically recover head-to-tail motor coordination after SCI; but only in warm temperature (23 ^{0}C), in cold temperature (10 ^{0}C) their coordination is most often abnormal or maladaptive. We used a comparative histology analysis in warm and cold environments, to examine the internal structure of larvae lampreys at 1 and 2 weeks during the regeneration process.

One week after SCI, blood clotted at the injury site in cold animals but not in warm. In warm animals, the dorsal and ventral nerve cut-ends had begun to regenerate. Two weeks after SCI, the cut-ends of warm animals have regenerated but only the dorsal and ventral ends, leaving a lagoon like opening on the center of the cord. In cold temperature, the blood clot persisted at the injury site and the cut-ends showed almost no sign of regeneration. These results showed that in larvae lampreys, warm temperature enhanced the ability to clear the injury site and regenerate the spinal cord, which was correlated to the more adaptive recovery at this temperature. These results

help us understand the factors that lead to a more adaptive recovery and contribute to the engineering of future therapeutic approaches for spinal cord regeneration.

2.1.2 Animal surgery and recovery

Larvae lamprey *(Petromyzon Marinus)* were anesthetized with 100 mg/ml MS-222 (Tricaine MS-222, Argent Labs, Redmond, WA). The animal was placed dorsal side up and the musculature opened with a longitudinal incision at mid-body using a surgical blade. The spinal cord was exposed using a pair of tweezers and completely transected using a surgical blade, carefully avoiding damage to any adjacent tissue. The muscle was sutured using a 6-0 suture (EP8889H, Ethicon, New Brunswick, NJ). During the effect of anesthesia the animals were kept under watch in ice water. After the anesthetic effect was gone, the animals were translated to aquariums in temperature controlled rooms set at two conditions: i) 20 animals were placed at 10-12° C and ii) 20 animals were placed at 20-22° C.

From each of these groups, half of the animals were sacrificed at 1 week and the other half at 2 weeks. The tissue fixed in 4% paraformaldehyde (Paraformaldehyde, Sigma-Aldrich, St. Louis, MO), for a total of 10 animals for each temperature and time point. The temperature and health of the animals were check daily, and they were fed brewer's yeast weekly. Animal maintenance and surgical procedures were approved by the University of Maryland's Institutional Animal Care and Use Committee, IACUC.

2.1.3 Histology, immunohistochemistry and in vivo imaging

At their respective time points (1 or 2 weeks) animals were sacrificed and placed into 4 % paraformaldehyde for 48 hours. For histology, a section of 3 cm from mid-body was cut and placed in a tissue processor (Leica TP1020) for 12 hours. The sample was embedded in paraffin (Leica EG1160), sagittal sections of 20-30 μm were obtained in a microtome (Microm HM 355 S) and 3 continuous tissue cuts were placed in a glass slide. The slide was left to dry overnight, then deparaffinized using HistoClear (National Diagnostics, Atlanta, GA) and stained using toluidine blue (Sigma-Aldrich, St. Louis, MO).

For immunostaining, a section of 3 cm from mid-body was cut and placed into mounting medium at -80 ^{0}C for 10 minutes. Frozen molds were placed into a cryotome at -20 ^{0}C, sagittal sections of 20-30 μm were obtained and 3 continuous tissue cuts were placed into slides. The slides were stained for neurofilaments (SMI-31 monoclonal antibody, Covance, Princeton, NJ) and nuclei (Hoechst, Sigma-Aldrich, St. Louis, MO). Microscopy images were obtained for each series of slices and analyzed using ImageJ (U. S. National Institutes of Health, Bethesda, MD). For analysis, we divided the spinal cord into 3 areas: upper, middle and lower and the surrounding meninges into dorsal and ventral. We measured the distance between the cut-ends for each area and calculated the average and standard deviation. The same images were analyzed by an independent source to ensure reproducibility of the measurements.

2.2 Adaptive vs. maladaptive regeneration

2.2.1 Control Animals: Histology and Immunostaining

In order to better understand adaptive vs. maladaptive regeneration, we characterized the uninjured animal first. We sectioned uninjured animals at sagittal and cross sectional planes and stained the tissue using histology or immunostaining. We stained paraffin sections using toluidine blue (TB), which revealed the spinal cord, meninges and other physical structures such as the notochord (Figure 2-1 a, c and g). When polarized, TB stained tissue highlighted particular structures, such as the collagen in the notochord and the borders of the spinal cord and the meninges (Figure 2-1 b, d). We used the spinal cord and meninges as our main points of reference for the comparison between animals regenerating in warm and cold temperatures.

Immunostaining was used to reveal more specific structures, such as the neurofilaments in the spinal cord (Figure 2-1 f) and cell nuclei (Figure 2-1 e). Once the structures in sagittal and cross sectional planes of control lampreys were characterized, we performed a comparative study of animals regenerating in warm and cold temperatures for 1 and 2 weeks.

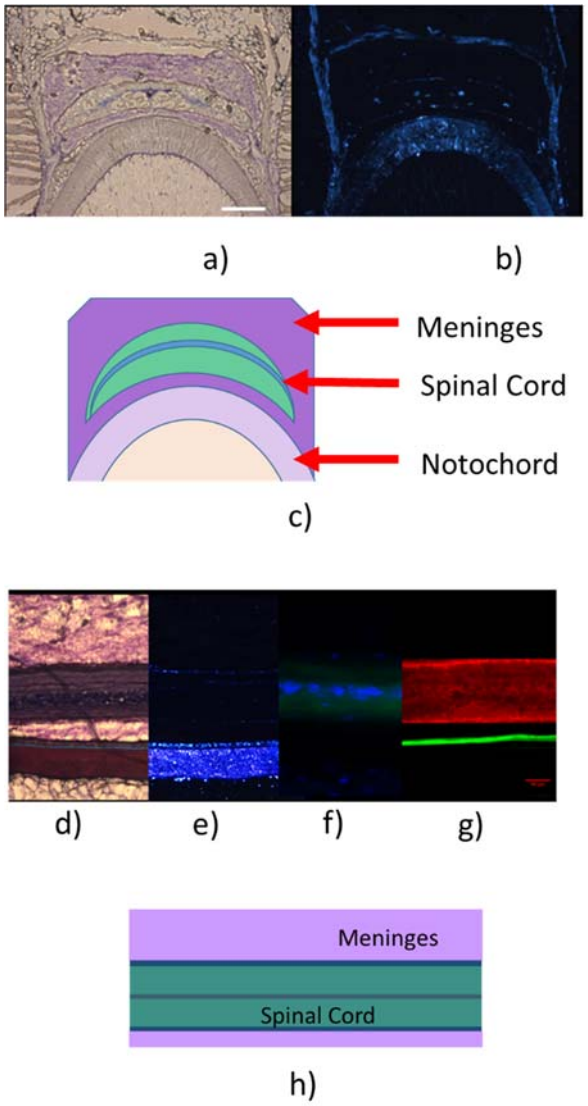


Figure 2-1. Cross sectional and mid-sagittal sections of the uninjured larvae lamprey. a) Cross section of the spinal cord and meninges at mid-body, stained using toluidine blue, scale bar is 100 µm b) Same cross section using polarized microscopy, highlighting the collagen in the notochord and the borders around the spinal cord. C-f) Control sagittal, from Left to Right. Toluidine Blue Histology (Meninges, Spinal Cord and Notochord), Polarized Microscopy, Nuclei (Hoechst Strain, Blue), Neurofilaments (SMI-31, Green) scale bar is 50 µm. g) Diagram of the structures that will be analyzed: spinal cord (blue) and surrounding meninges (purple).

2.2.2 Adaptive vs. Maladaptive: 1 week after SCI

After complete transection of the spinal cord at mid-body, we left the animals regenerate for 1 week and then sectioned the tissue at the sagittal plane. We observed the separation of the nerve cut-ends and the meninges (Figure 2-2 b), followed by the presence of blood cells at the injury site (Figure 2-2 d). These cells appeared to concentrate at the empty space in the injury site but also close to the spinal cord (Figure 2-2 b, c), potentially blocking the regeneration of the cut-ends.

Comparison of animals in cold and warm temperature revealed two clear differences: the amount of blood at the injury site and the distance between the cut-ends. In Figure 2-3 a-b, we can observe 2 different animals that were characteristic of regeneration in warm temperature. In one of them the spinal cord was not regenerated but there was significantly less blood at the injury site, compared to cold animals (Figure 2-3 a). Thus, potentially leaving a free physical pathway for axons to regenerate. For the other warm animal (Figure 2-3 b) there was already regeneration between the ventral ends of the spinal cord, which showed the enhanced growth rate in warm temperature compared to cold.

The injury site of cold animals at 1 week was characterized by an increased number of blood cells at the injury site that could be a physical barrier for axonal regeneration (Figure 2-3 c-d). Furthermore, the gap between the cut-ends of the spinal cord and the meninges (Figure 2-4 b) was larger than that of warm animals (Figure 2-4 a). Overall, at 1 week after injury, the lower part of the spinal cord in warm

temperature was able to regenerate (Figure 2-4 a), while in cold temperature regeneration seemed to be blocked by a large number of blood cells (Figure 2-4 b).

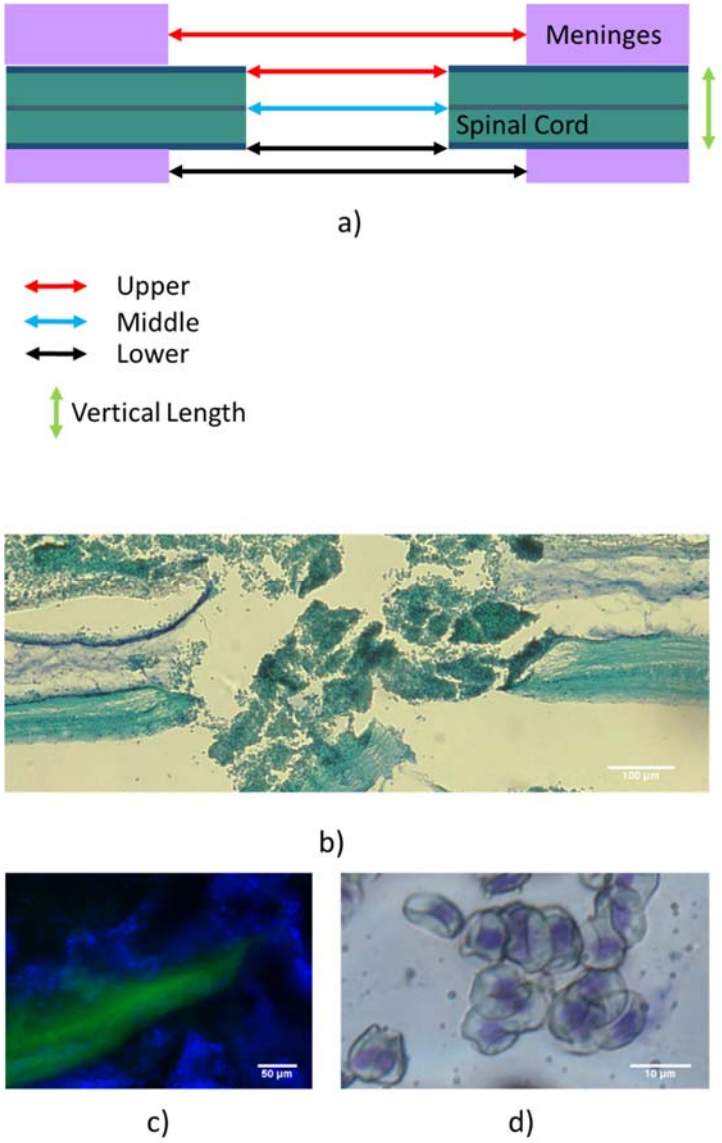


Figure 2-2. The injury site after spinal cord injury. a) Diagram of the tissue after spinal cord injury, including the meninges (purple) and spinal cord (blue). The arrows indicate the distance between the cut ends at the different sections: upper, middle and lower. The green arrow indicates what we defined as vertical length. b) Sagittal section of the injury site stained with toluidine blue, c) Immunostaining section of the nerve cut-end, stained with SMI-31 (green) for neurofilaments and Hoechst (blue) for cell nuclei of

blood cells present at the cut-ends. d) Magnified section of the blood cells that form the clot at the injury site, stained with toluidine blue.

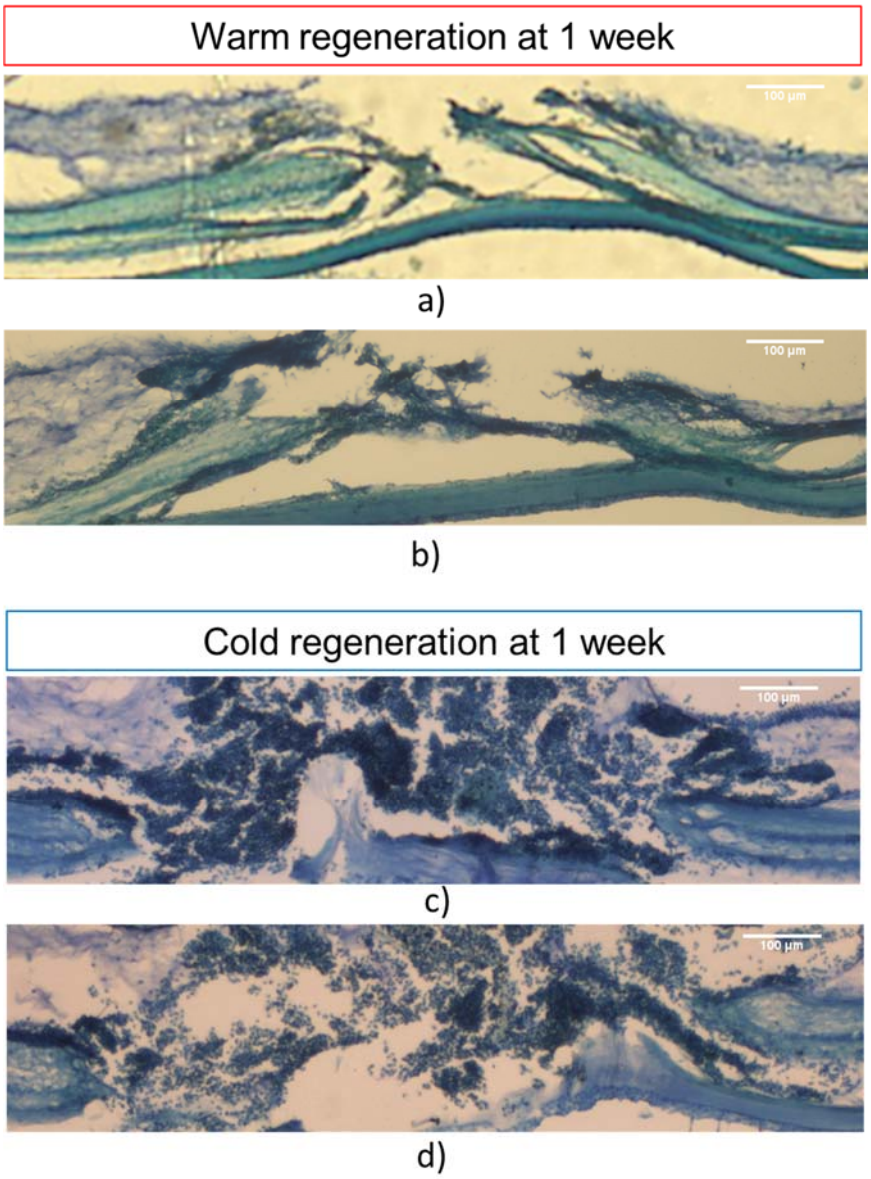


Figure 2-3. Sagittal sections after 1 week of regeneration. a-b) Sagittal sections of two different animals 1 week after SCI regenerating in warm temperature. c-d) Sagittal sections of two different animals regenerating in cold temperature. Sagittal sections of 10 animals were obtained but not all of them contained the upper, middle and lower areas. Scale bar is 100 µm for sagittal sections and 50 µm for the cross sections.

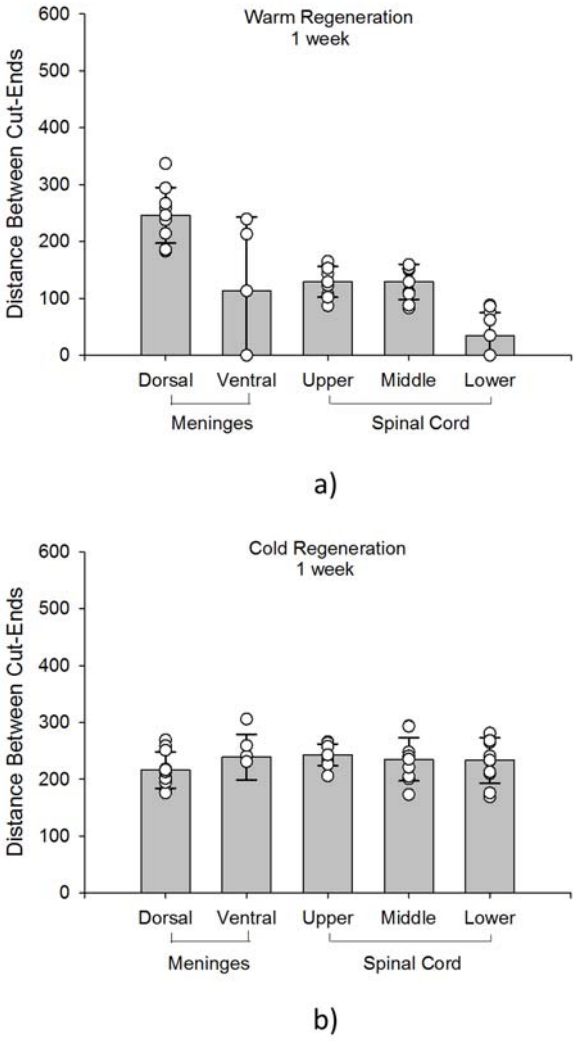


Figure 2-4. Distance between the cut ends at 1 week. a) Distance between the cut-ends of meninges and spinal cord from animals in warm temperature, bar plots indicate the average and white circles indicate the value of each animal. b) Distance between the cut-ends of animals regenerating in cold temperature. Error bars indicate the standard deviation, sagittal sections of 10 animals were obtained but not all of them contained the ventral meninges. Only 4 animals had ventral meninges in warm temperature and only 3 had them in cold temperature.

2.2.3 Adaptive vs. Maladaptive: 2 weeks after SCI

After 2 weeks of regeneration, the differences between warm and cold environments were remarkable. Warm animals have regenerated the spinal cord (Figure 2-3), but only the outer upper and lower sections (Figure 2-5 a-c). Most animals regenerated the lower section (Figure 2-5 a, c), while others have regenerated both sections, leaving a lagoon-like opening on the middle of the spinal cord (Figure 2-5 b). Cross sectional staining confirmed that the regenerated tissue had neurofilaments (Figure 2-5 d, e) and most of the regeneration was located on the outer sections.

Animals left to regenerate in cold temperature showed little or no regeneration at 2 weeks (Figure 2-6). Figure 2-6 a-c demonstrates 3 different animals in cold temperature, for all of them the blood clot was still present at the injury site. This provided further evidence that the blood clot could be a reason regeneration in cold temperature is maladaptive. Cross sections at the nerve cut-ends revealed the presence of neurofilaments (Figure 2-6 d), but the spinal cord seemed to have lost its clear elliptical shape (Figure 2-1 a). Immunostaining at the injury site revealed the presence of only cell nuclei, most likely from blood cells that formed the clot (Figure 2-6 e).

We measured the gap between the meninges above and below the spinal cord (Figure 2-2 a). Surprisingly, regeneration of the meninges in warm temperature was no better than that in the cold (Figure 2-7 a, b). When compared over time, the meninges didn't regenerated significantly and in some animals the gap increased. This effect was

more obvious in the ventral meninges at cold temperature, where the average gap was significantly larger at 2 weeks compared to 1 week.

Then, we measured the gap between the upper, middle and lower ends of the spinal cord (Figure 2-2 a). For warm animals, the upper and lower ends of the spinal cord regenerated but not the middle of the cord. In fact, the middle cord did not change over time. For the upper ends, 4 animals out of 10 regenerated and for the lower ends 9 animals out of 10 regenerated (Figure 2-7 a). Cold animals didn't show any regeneration or difference between the upper, middle and lower ends (Figure 2-7 b).

In addition to horizontal changes, we noticed that the vertical length of the spinal cord increased close to the injury site (Figure 2-8 a). We used polarized microscopy (Figure 2-8 b) to measure the vertical length of the cord 3 mm after and 3 mm before injury, being "after" the caudal part and "before" the rostral part of the animal. At 1 week, in warm temperature the vertical length of the cord larger at the injury site, and it decreased until reaching a normal length 1 mm away from the injury (Figure 2-8 c). In cold temperatures, the vertical length didn't show any changes (Figure 2-8 c). At 2 weeks, the vertical length of the cord in cold animals increased (Figure 2-8 d) but the cut-ends showed no signs of horizontal regeneration. In comparison, the regenerated area of warm animals had a vertical length larger than regions far from the injury (Figure 2-8 d). Interestingly, at 2 weeks the vertical length of the cord at the injury site was the same in warm and cold temperature.

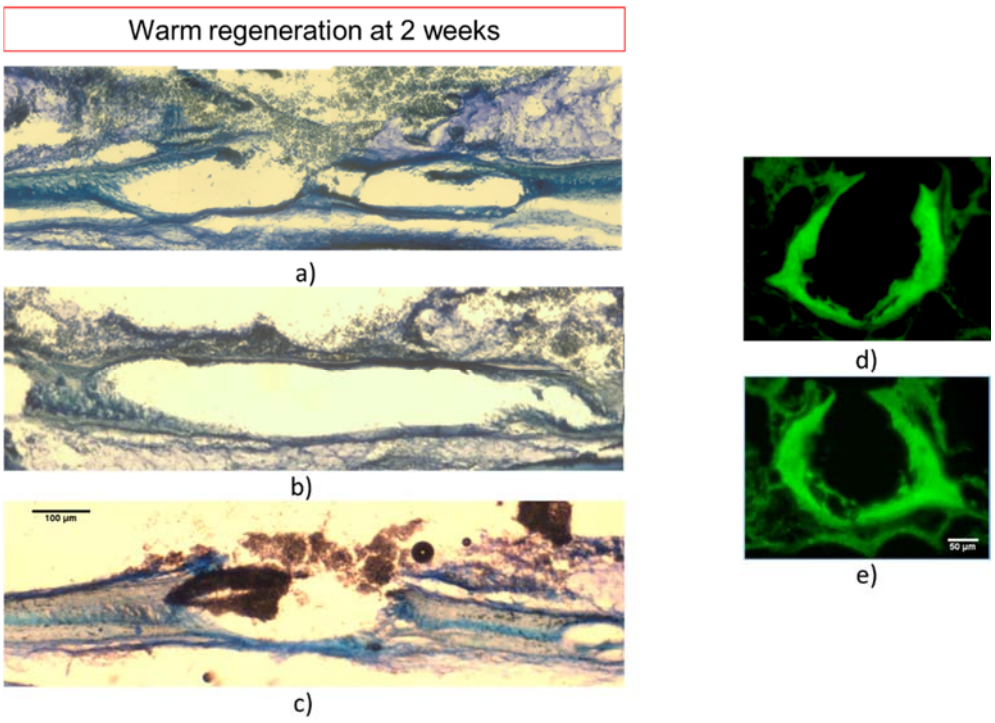


Figure 2-5. Sagittal and cross sectional images of warm regeneration at 2 weeks. a-c) Sagittal sections of 3 different animals, a) Animal that has regenerated only in the bottom, but the upper part has significantly grown, b) Animal that has regenerated both upper and bottom parts of the spinal cord, c) Animal that has only regenerated the bottom and the upper part has not grown. d-e) Cross sectional views of the regenerated spinal cord stained with SMI-31 (green) for neurofilaments, showing that only the outer parts of the spinal cord express neurofilaments. Scale bar is 100 µm for sagittal sections and 50 µm for the cross sections.

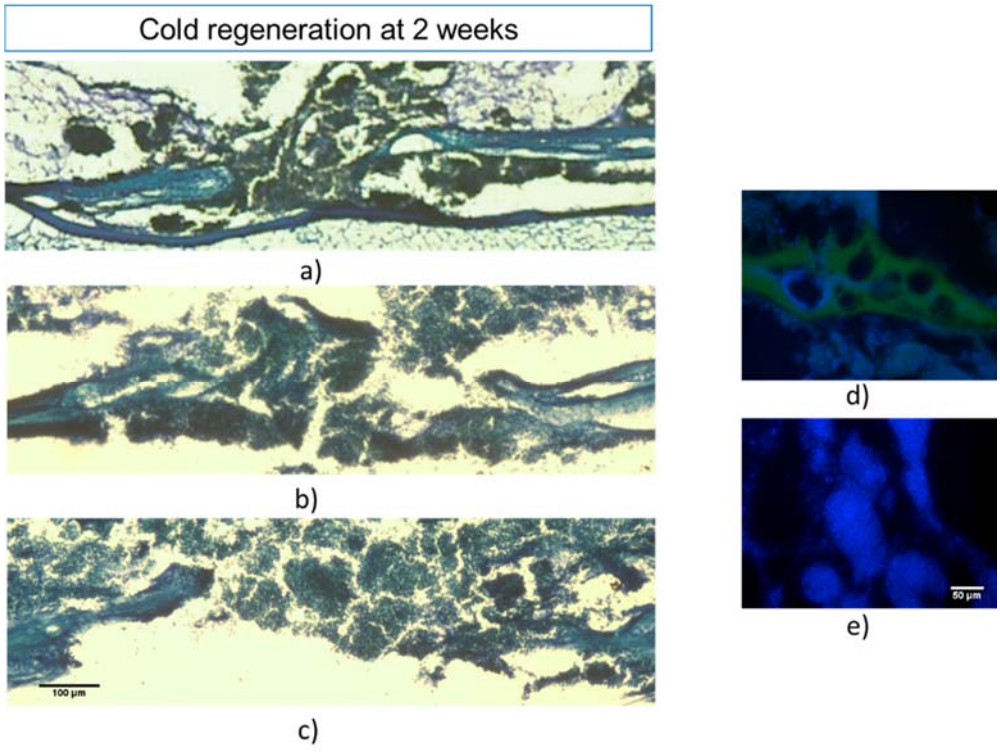


Figure 2-6. Sagittal and cross sectional images of regeneration in cold temperature. a-c) Sagittal sections of 3 different animals that show the clot at the injury site and the lack of spinal cord regeneration. d-e) Cross sectional images stained with SMI-31 (green) and Hoechst (blue) to show cell nuclei. d) Cross section at the nerve end that shows the end of the spinal cord, e) Cross section at the injury site that shows the lack of SMI-31 expression and the content of only cell nuclei. Scale bar is 100 µm for sagittal sections and 50 µm for the cross sections.

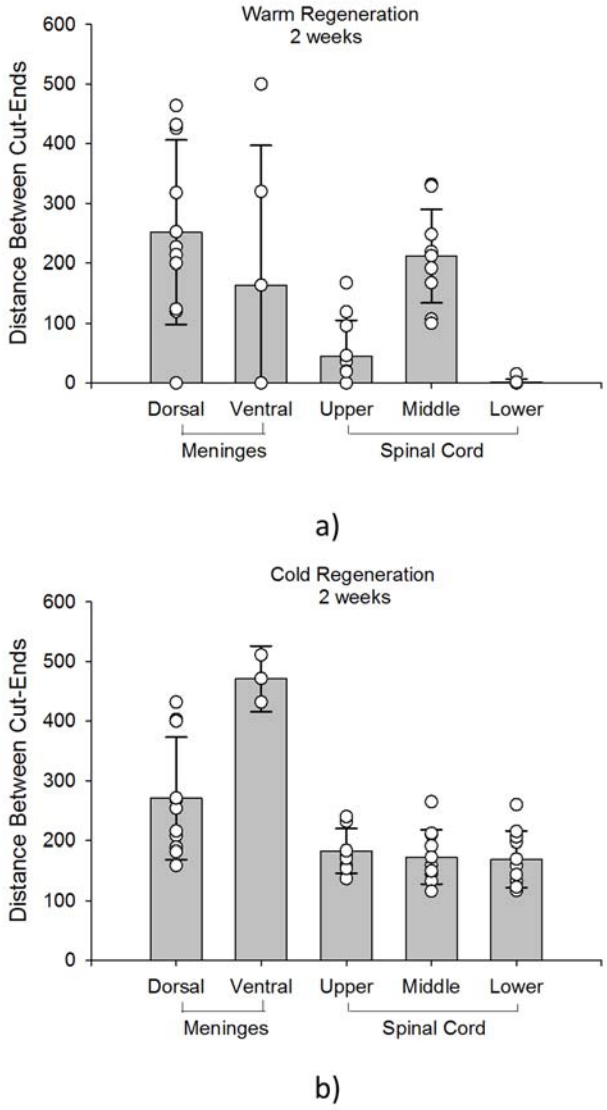


Figure 2-7. Distance between the cut-ends after 2 weeks of SCI. a) Distance between the cut-ends of animals regenerating in warm temperatures, note that some of the animals have regenerated the bottom of the spinal cord and some the upper part, but not the middle. b) Distance between the cut-ends of animals regenerating in cold temperature. Note that the upper, middle and bottom sections of the spinal cord have not regenerated and have the same average gap at the injury site. N=10. Only 6 animals had ventral meninges in warm temperature and 3 animals in cold temperature.

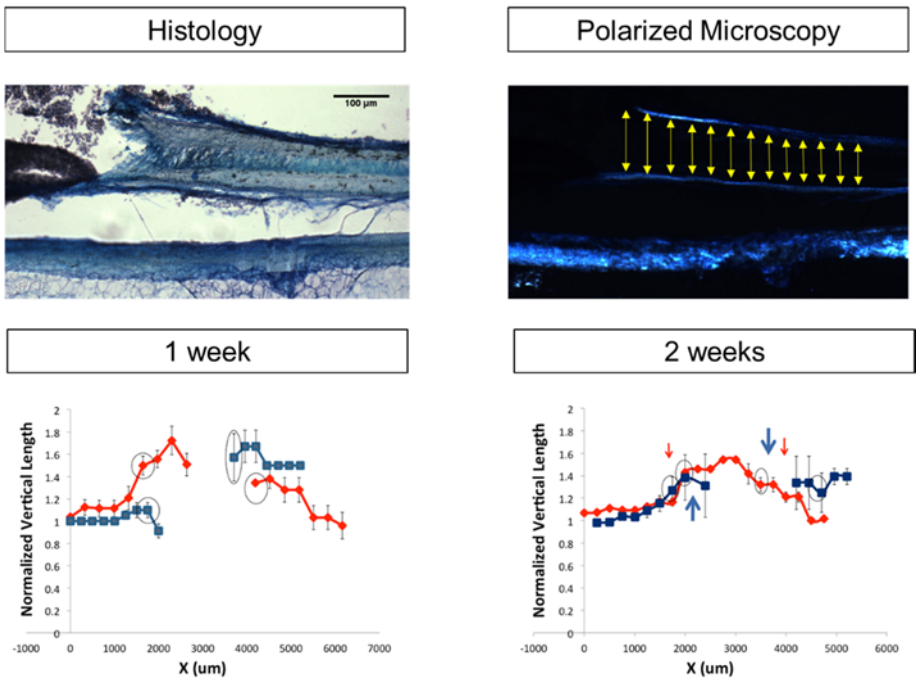


Figure 2-8. Changes in the vertical length after SCI. a) Sagittal section stained with Toluidine blue, showing the nerve-cut and how it changes close and far away from the injury site, b) Same section using polarized microscopy showing the borders of the spinal cord and the place where the measurements of the vertical length were taken. c) Vertical length for warm (red) and cold (blue) regeneration at 1 week, note the warm regeneration has an increase vertical length close to the injury site (circles indicate the average location of the meninges) d) Vertical length for warm and cold regeneration at 2 weeks, warm animals who have already regenerated maintain an increased vertical length throughout the regenerated site. At this time point, cold animals have actually increased their vertical length to levels similar to that of warm, the only difference was the lack of reconnection. N=10, scale bar is 100 µm for all figures.

2.3 Summary and Conclusions

2.3.1 Summary

In this work we compared the structural differences after SCI for animals regenerating in warm (adaptive) and cold (maladaptive) temperature (Figure 2-9). Through this comparison, we were able to better understand factors that could influence the functionality of regeneration. The first remarkable difference was the amount of axonal regeneration in warm temperature at 2 weeks, while cold animals have not even started regenerating (Figure 2-9 b, c). Regeneration was enhanced, but unable to recover the full structure of the uninjured spinal cord, as only the outer areas regenerated. However, a fully regenerated spinal cord might not be needed to recover behavior since it has been shown that lampreys only need few and small synapses to recover motor coordination [61]. Our results seem to indicate that the middle of the spinal cord, which contains mainly cell nuclei (Figure 2-1 f) and giant axons doesn't regenerate within 2 weeks. This is an important result, as it shows evidence that the spinal cord regenerates mainly the propiospinal neurons and not cell nuclei or giant fibers. Our results coincide with those from other researchers who have labeled the ascending and descending regenerating spinal projection neurons [143] and neurites emanating from injured axons, distal and proximal to the scar [76]. They have found that the regenerating neurons project their axons up to 5 mm in both ascending and descending directions across the injury site, but not the cell bodies.

2.3.2 Meninges

Although it has been stated that meninges are necessary for spinal cord regeneration on animals, such as the newt [56], we didn't observe this phenomenon in the lamprey at least up to 2 weeks after injury. However, we observed that the meninges in cold temperature had a wider gap than in warm. They seemed to have degenerated from 1 week to 2 weeks. Degeneration of meninges in cold temperature could cause maladaptive behavior, since their function is to protect the spinal cord [144-146]. Meninges might also harbor niches for precursor cells that help regeneration, which could make their presence close or at the injury site important for adaptive regeneration [146, 147]. The lack of meninges surrounding the spinal cord at the injury site could also be one of the causes for the vertical length to increase (see circles in Figure 2-8).

2.3.3 Adaptive vs. Maladaptive: Blood cells and temperature

Another key difference between adaptive and maladaptive animals was the amount of blood cells at the injury site. Therapeutic approaches to control the immune response are among the most important for spinal cord injuries [148], and our research provides even more evidence of their key role. We showed that when clotted, these cells could be a barrier for axonal regeneration and contribute to the maladaptive recovery in cold temperature (Figure 2-9 b). Warm animals showed an accelerated recovery, the injury site was free of a clot and the cord regenerated (Figure 2-9 c). It remains to be understood the dynamic behavior of the injury, and the real-time mechanism through which immune cells move away from the injury site in warm temperature.

The effects of temperature on spinal cord injuries have been observed for a variety of animals. In turtles, warm environments (27-30 ^{0}C) increased cell proliferation in the CNS compared to colder environments (5-14 ^{0}C) [149]. Similarly, on the weakly electric fish, spinal cord regeneration at high temperatures (30 ^{0}C compared to 20 ^{0}C) was twice faster than normal [65]. A different approach has been used in animals that don't regenerate, such as rats [150-153] and humans [5, 154, 155], where hypothermia treatments (from 37 ^{0}C to 32 ^{0}C) have been explored because they might attenuate the inflammatory response after SCI and reduce secondary damage, but these effects still remain controversial [155, 156].

Lampreys and humans control their temperature differently, and their normal temperature lies in a different range (10 ^{0}C and 37 ^{0}C) thus, the ranges at which temperature can be varied and the effects on the organism will vary greatly. However, the basic principles of cell proliferation at increased temperatures and the attenuation of the immune response at low temperature seem to be similar. Lampreys regenerating in cold temperatures have what appears to be a "slow-down" immune response leaving the blood clotted at the injury site, and in the long term this might lead to a maladaptive behavior. In human spinal surgeries, lengthy exposure to hypothermic conditions has been associated with complications such as infection [156]. On the other hand, warm temperatures could enhance the proliferation of human cells, as it has been observed with bone marrow derived stromal cells [157], however fever and hyperthermia are a great problem for patients with spinal cord injuries [158-160].

In fact, thermoregulatory dysfunction is a highly common problem after SCI [160, 161]. Thus, making a controlled study of temperature changes in humans after SCI a highly complex problem. The results showed here demonstrate that the lamprey could be an efficient model to study changes in temperature as well as differences in the immune response and axonal regeneration. It provides a very clear model for maladaptive and adaptive behavior and could be a successful animal model to study therapeutic approaches. For example, researchers could study the effects of surgical approaches or drugs, such as chondroitinase ABC and how they can improve or worsen maladaptive regeneration.

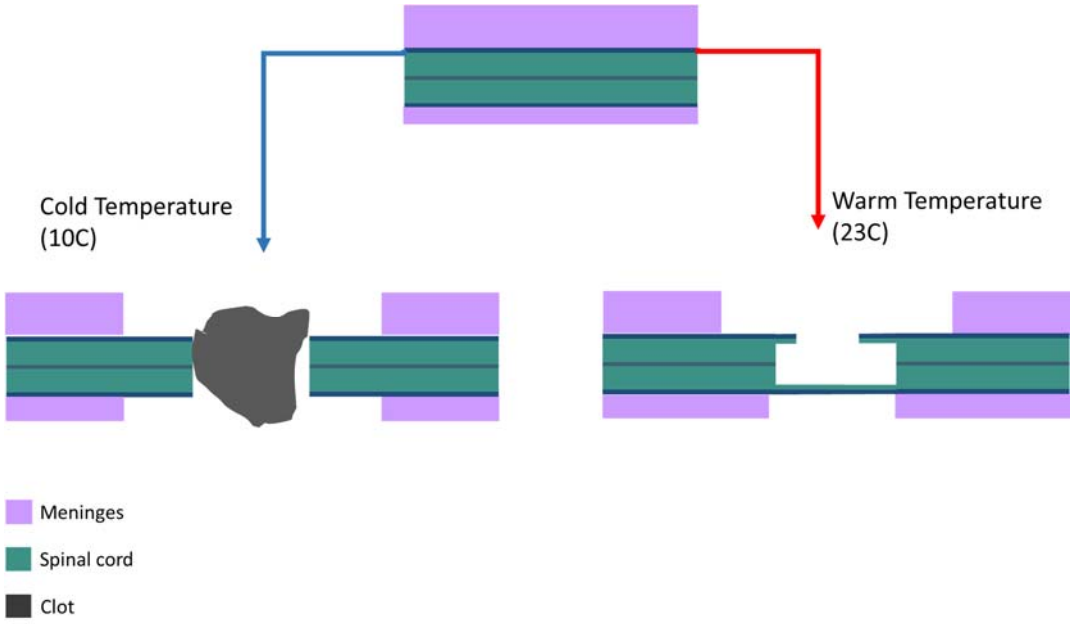


Figure 2-9. Diagram of cold and warm regeneration. After complete spinal cord injury, both the meninges and spinal cord separate and the animal loses functionality. When the animal regenerates in cold temperature (Left), a clot formed that seemed to block regeneration. Surprisingly, the same animal model in warm temperature didn't have a clot and the upper and middle areas of the spinal cord regenerated, but not the middle part of the cord or the surrounding meninges, in the time we gave them to regenerate (2 weeks).

Chapter 3: Imaging, structure and biomechanics of adaptive and maladaptive regeneration

3.1 Experimental Methods and Rationale

3.1.1 Rationale

During spinal cord injury, nerves suffer a strain beyond their physiological limits which damages and disrupts their structure. Research has been done to measure the modulus of the spinal cord and surrounding tissue; however the relationship between strain and spinal cord fibers is still unclear.

In our studies we employed larval lampreys, a jawless vertebrate that has been used for genetics [162, 163], animal locomotion [164-167] and spinal cord regeneration [61, 78, 168, 169]. Sequencing their genome has provided insights into the principles and evolution of vertebrate biology [162]. Lampreys have also been shown to recover locomotion after complete spinal transection [78], which makes them an important model for spinal cord injury and regeneration [80]. However, the mechanisms to achieve complete and adaptive regeneration remain elusive. The notochord, the axial support of the organism, can reasonably be expected to play a role in normal animal locomotion and recovery after injury. However, the role of the notochord has remained unexplored. With the use of *in vivo* imaging we are now able to fill this void. We show here, detailed information regarding the structure and properties of the notochord.

3.1.2 In vitro and In vivo Tensile loading testing

Uniaxial loading experiments were carried out using a custom-made set up (Figure 3-1 a) that consists of a set of micromanipulators (Model M-460A-xyz and SM-13, Newport, Montana, USA) that controlled the overall tissue displacement and a force transducer (Force Range 0 to 10.0 mN, Sensitivity 1.0 mN, Resolution 200.0 nN, Linearity ±0.2% of full scale over 50% of full scale, ±1.0% of full scale over full scale, Maximum Overload Force 100.0 mN, Model 405A, Aurora Scientific, Ontario, Canada) . Freshly isolated spinal cords (3-5 cm long) were placed in a chamber filled with Lamprey saline solution (58.44 g/mol NaCl, 74.55 g/mol KCl, 110.98 g/mol CaCl2, 203.3 g/mol MgCl26H20, 238.3 g/mol HEPES, 180.2 g/mol Glucose, Sigma Aldrich, Missouri, USA), to avoid dehydration, and gripped using surgical grade fibers to both wires connected to the micromanipulator and force transducer respectively (Figure 3-1 b).

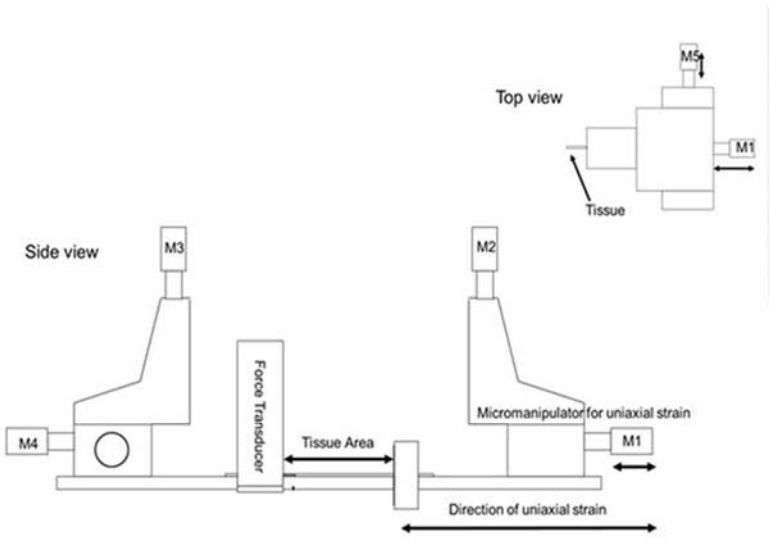
The gripping method was modified from [170], a double overhand suture loop was used to secure the tissue without slippage; while fibers were compressed at the knot, the absence of breaks or slippage allowed consistent behavior from trial to trial. Readings were taken in the steady state; ~ 10 min after the spinal cord was stretched approximately 0.5 to 2 mm at a time using the manipulator. Each force value (F) was then divided by the area of the spinal cord (A) to obtain the stress ($\sigma = \frac{F}{A}$). For the stress-strain calculations, the spinal cord was assumed a cylinder. The diameter was measured using a dissection microscope, every strain value the diameter was measured to obtain the area for each point. The strain was obtained by dividing the change in

spinal cord length (L-L_o) over the original length (L_o), ($\varepsilon = \frac{L-L_0}{L_0}$). The results of 10 spinal cords without meninges, 5 samples for each section (Head, Middle and Tail) and 5 cords with meninges were plotted as a stress vs. strain curve. Statistical analysis was performed using Student's t-test and factorial analysis of variance (ANOVA) with a $p < 0.05$.

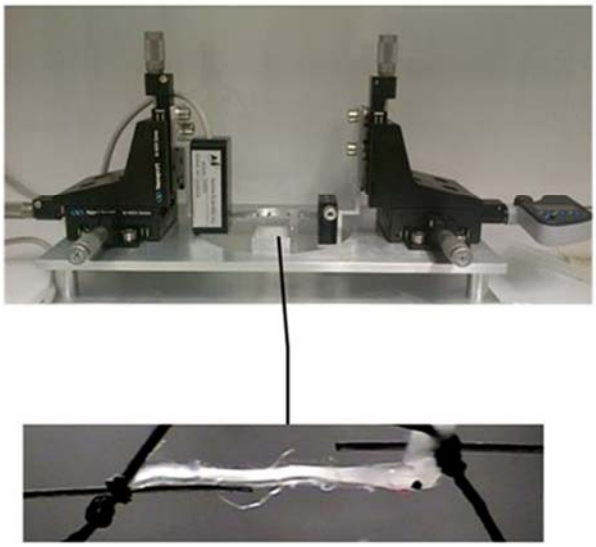
In vivo experiments were made by first anaesthetizing the animal with MS222 (100 mg/l). The spinal cord was exposed and the musculature pinned open, but not removed. Two polyester glint marks were placed at the start and end of the cord and the distance between them measured. Then, both points were cut with a scalpel and the shortened cord was measured, this experiment was repeated for 5 different animals and the physiological uniaxial strain obtained.

Similarly, in order to understand the physiological threshold levels, we repeated this experiment by placing the animal into the most common swimming position. The swimming position was defined when the body had two areas of maximum curvature, each localized at approximately 25% and 75% of its own length. The animal was anesthetized, the musculature pinned open and marks are placed in the spinal cord. We placed two marks separated approximately 1 cm in the Head section (below the gills), two marks at the Middle section (3 cm below the Head section) and two marks at the Tail section (3 cm after the dorsal fin).

First the animal was placed straight and the length between each marker was measured. Then the animal (while anesthetized) is moved into the swimming position and the longitudinal length between each marker was measured and the local uniaxial strain is calculated. Even when anesthetized, the animal will adopt the swimming position almost automatically, thus the amount of force needed to bend the animal is minimal. Statistical analysis was performed using Student's t-test and factorial analysis of variance (ANOVA) with a $p < 0.05$.



a)



b)

Figure 3-1. Tensile loading apparatus. a) Design of the custom made tensile loading apparatus which consists of a set of micromanipulators to control x,y and z directions and a force transducer to measure the force response, b) Custom made apparatus and a magnification of the gripping method of the spinal cord during uniaxial longitudinal strains

3.1.3 Mathematical modeling

In order to understand the effect of longitudinal fibers on the uniaxial strain, we decided to use a theoretical approach. Consider a composite tissue formed by unidirectional fibers (A_f) embedded in a matrix (A_{mtx}) (Figure 3-2) with total cross-sectional area ($A_{total} = A_{mtx} + A_f$). The tissue is subjected to a uniaxial load in the longitudinal direction ($\sigma = \frac{F}{A_{total}}$); assuming that both the matrix and the fibers have a linear behavior in the low strain and high strain regions ($\sigma = E\varepsilon$).

When strains are too low the stress response is small thus we can assume that all the fibers have slack (having very low stress) and their contribution to the total stress can be ignored. Therefore the load on the total material is mostly due to the matrix stress, which can be expressed by:

$$\sigma = \begin{cases} \overline{A}_{mtxH} E_{mtx} \varepsilon & (1) \\ \overline{A}_{mtxM} E_{mtx} \varepsilon & (2) \\ \overline{A}_{mtxT} E_{mtx} \varepsilon & (3) \end{cases} \qquad \varepsilon < 0.2$$

where $\overline{A}_{mtxH} = \frac{A_{mtxH}}{A_{totalH}}$ is the area fraction of the matrix (area of matrix divided by the total area of the spinal cord), the H, M and T indicates the corresponding section (Head, Middle or Tail). Assuming that the matrix is the same throughout the body, the modulus E_{mtx} is the same for all sections.

At higher strains when the fibers are straightened, their contribution to the total stress becomes significant and using the equistrain rule of mixtures [171, 172] we can express the load applied to the tissue as:

$$\sigma = \begin{cases} (\overline{A}_{fH}E_f + \overline{A}_{mtxH}E_{mtx})\varepsilon & (4) \\ (\overline{A}_{fM}E_f + \overline{A}_{mtxM}E_{mtx})\varepsilon & (5) \\ (\overline{A}_{fT}E_f + \overline{A}_{mtxT}E_{mtx})\varepsilon & (6) \end{cases} \qquad \varepsilon > 0.2$$

where $\overline{A}_{fH} = \frac{A_{fH}}{A_{totalH}}$ is the area fraction of the fibers (area of fibers divided by the total area of the spinal cord), the H, M and T indicates which section the area corresponds (Head, Middle or Tail). If we assume that all the fibers have the same tensile modulus then E_f is the same for all equations.

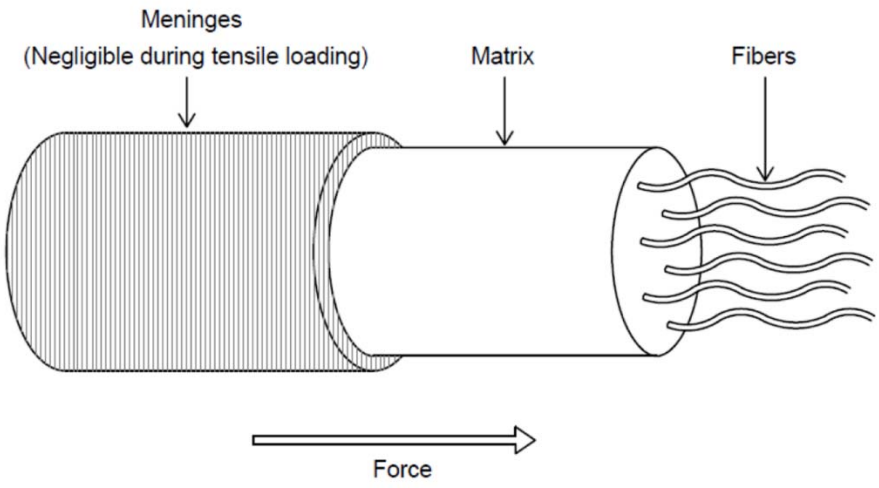


Figure 3-2. Schematic representation of the spinal cord elements considered in the composite-material model. This figure shows a simplified model of the spinal cord for theoretical analysis that considers the basic elements that are significant during tensile loading: the matrix and fibers parallel to the tensile load.

3.1.4 Histology and Immunostaining

To better understand the uniaxial strain response of different sections in the spinal cord, we used histology to measure the number of large fibers at the Head, Middle and Tail sections. Spinal cords were fixated using Mirky's Fixative solution (National Diagnostics, Georgia, USA), following dehydration in ethanol series; Head, Middle and Tail sections were embedded in paraffin and a series of 5 (20 μm thick) slices obtained and stained with toluidine blue (Sigma Aldrich, Missouri, USA). Microscopy images were obtained for each series of slices and large fibers [173] were identified and the number measured using ImageJ (U. S. National Institutes of Health, Maryland, USA).

Fresh spinal cords, with and without meninges were stained using fluorescent wheat germ agglutinin (WGA, Molecular Probes, Oregon, USA). After isolation from the body and removal or not of the meninges, spinal cords were incubated for 5 min at 4°C in 10 μg/ml concentration of WGA. The spinal cord was washed twice in HBSS solution and incubated for 5 min at 37°C in HBSS.

3.1.5 In vivo X-ray phase-contrast imaging (XPC) and polarized microscopy

X-ray imaging at a synchrotron source offers new possibilities for biological research with the ability to image soft tissue in vivo [174] avoiding the complications associated with contrast agents [175], as well as the anatomical and mechanical disruptions associated with dissection of the intact animal. Through the internal visualization of tissue physiological dynamics, researchers have discovered the

respiratory movements of the trachea of insects [176]. XPC with its impressive temporal and spatial resolution also allows for the visualization of biological tissue [174], cancer lesions [177], animal physiology [178] and anatomical details of air-containing organs [179, 180].

Larval lampreys were anesthetized using tricane (MS-222, 100 mg/ml) for 30 minutes before each imaging session. They were placed in a polyethylene container filled with water, which tightly surrounded the body of the animal. The chamber was clamped and suspended in the pathway of the partially coherent synchrotron x-ray beam and phase contrast imaging was observed using 20 keV photons (Figure 3-3 a). Experiments were carried out at XOR-32ID at the Advanced Photon Source, Argonne National Laboratory with all the protocols approved by the animal care (Protocol R-10-39, IACUC, University of Maryland).

Polarized microscopy was used in combination with histology mainly to reveal the collagen fibers of the notochord. The sample was placed between two polarizing filters (polarizer and analyzer) and rotated to find the angle of maximum polarization (Figure 3-4). The image is produced from the interaction of plane-polarized light with a birefrigent tissue sample. After exiting the sample, the light components are out of phase and are recombined when they pass through the analyzer. This technique allowed us to obtain a high contrast image of the collagen fibers in the notochord tissue.

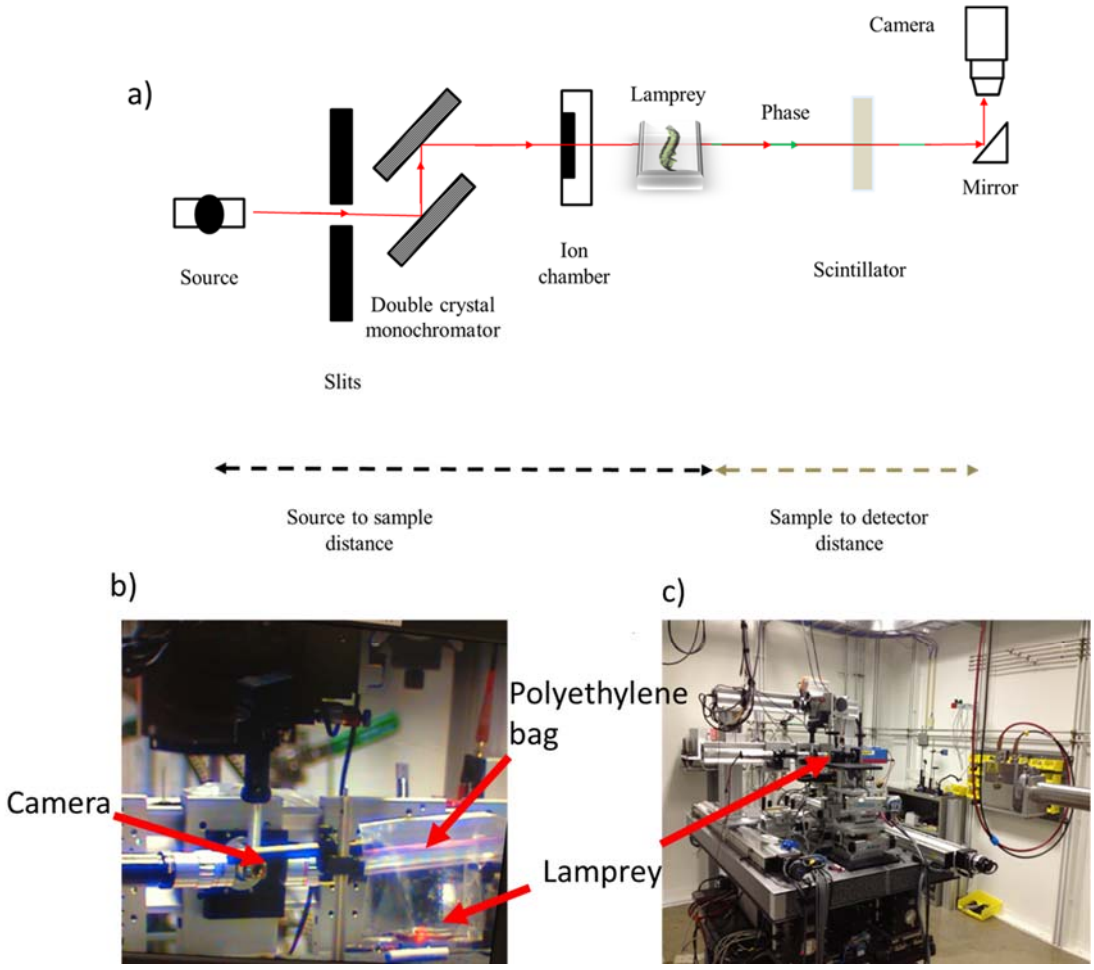


Figure 3-3. Set-up for X-ray phase contrast imaging. a) Schematic for x-ray phase contrast imaging, b) Sample holder, c) X-ray imaging station.

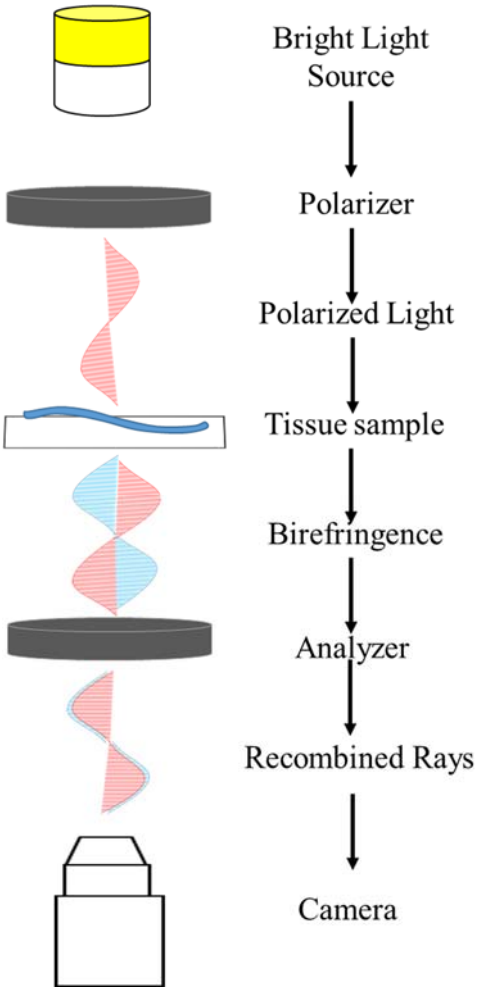


Figure 3-4. Polarized microscopy configuration.

3.2 Biomechanics of the spinal cord before and after injury

3.2.1 Tensile loading and modeling of an uninjured spinal cord

Using our custom tensile loading apparatus, we measured the strain-stress response of isolated spinal cords and different sections (Head, Middle and Tail). We found that the lamprey spinal cord behaves like a soft tissue material [181] (Figure 3-5 d) with a stress-strain curve characterized by two regions. The low strain region, ~ 0-18% strain has a modulus of ~0.015 MPa (Table 3-1). As the strain increases, the curve changes slope, reaching a higher strain region with a 0.5 MPa modulus (Table 3-1). The intersection between the low and high modulus, called the critical strain, was found to be an average 18% strain (Table 3-1).

After the cord was isolated from the body, we observed a contraction in length, which indicated the existence of a pre-stressed state. Using markers on the spinal cord, we found that the cord retracts an average of 10% of its original length. We considered this value as the physiological uniaxial strain at rest. Next, we analyzed the local uniaxial strains at the Head, Middle and Tail during swimming. We found local uniaxial strains at the Middle and Tail sections and almost no local strain in the Head section (Figure 3-5 c). If we add the total local uniaxial strain during swimming to the strain at rest, we find a maximum local uniaxial strain level of 15%. Therefore, we established the in vivo uniaxial strain levels between 10-15% as indicated in Figure 3-5 d.

Similar as for whole cord measurements, we divided the spinal cord into 3 sections (Head, Middle and Tail) in order to test the homogeneity of the mechanical

properties along the spinal cord. We measured the stress response to uniaxial tensile longitudinal strain and calculated the modulus for the low and high strain regions. We found that the low strain region is statistically equal for all, but the high strain region is different for the Tail compared to Head or Middle section (Table 3-1). The critical strain was found to be within 16-18 % strain (Table 3-1).

The stress values for the low strain region (0-18%) are statistically equal in all sections (Figure 3-6 a); however, at higher strains the stress response shows a decreasing trend from Head to Tail (Figure 3-6 a). These results indicate a difference in the material properties of spinal cord sections, which could come from differences in the number of fibers, matrix composition or meninges. Therefore, we decided to explore the effect of the meninges and the number of fibers.

We found that the response of cords with and without meninges was not different (Figure 3-6 b). To check we effectively removed the meninges, we used fluorescence staining (wheat germ agglutinin, Figure 3-6 c) on cords with and without the meninges. We can observe (Figure 3-6 b) that the meninges are successfully removed.

Using histology, we analyzed the total area of the spinal cord (Figure 3-7 a-b), and the number of large fibers between Head, Middle and Tail sections (Figure 3-7 c). The large fibers were measured using toluidine blue stained cross sections, we considered large fibers to be those with a radius larger than 20 μm. We found that the

total area decreases ~35% from Head to Tail, as can be observed in (Figure 3-7 b). Similarly, the number of large fibers decreases ~25% (Figure 3-7 c). These results indicate that the number of fibers can potentially affect the tensile response to high strains. To explore this effect, we adapted a mathematical model that correlates the modulus with spinal cord composition.

From the strain-stress data (Table 3-1) we know the value of the slopes at both, high and low strains, and from the histology data (Figure 3-7) we know the total cross-sectional area of the Head, Middle and Tail sections (Atotal). Therefore, we can solve the system of equations 1-6 to obtain Ef and Emtx. Using this model, the modulus of the large fibers is 2.4 MPa (Ef) and the matrix is 0.017 MPa (Emtx).

This mathematical model predicts a ~15% decrease in the area fraction of fibers from Head to Tail (Figure 3-8), which agrees with histological measurements that show a 12% decrease in the area occupied by the large fibers (Figure 3-8). Using the low and high modulus of different sections, we were able to approximate the fiber and matrix modulus, as well as changes in the concentration of fibers. Therefore, establishing a relationship between uniaxial strain and spinal cord composition that can be used in future studies with regenerated spinal cords.

	Low-strain linear region (MPa)	High-strain linear region (MPa)	Critical strain (%)
Whole cord	0.01578 ± 0.004	0.5 ± 0.1	18±5
Head	0.0165 ± 0.004	0.57 ± 0.3	16.6 ± 1.2
Middle	0.0185 ± 0.003	0.45 ± 0.2	17.5 ± 1.2
Tail	0.0176 ± 0.004	0.2 ± 0.1	18 ± 0.9

Table 3-1. Moduli for the whole spinal cord and different sections. The table shows the calculated modulus for the low-strain and high-strain linear regions using the stress-strain curves. Critical strain was calculated for the Whole cord, Head, Middle and Tail sections (critical strain is defined as the point where the stress-strain curve changes slope). Values are averages from 10 samples and error is the standard deviation.

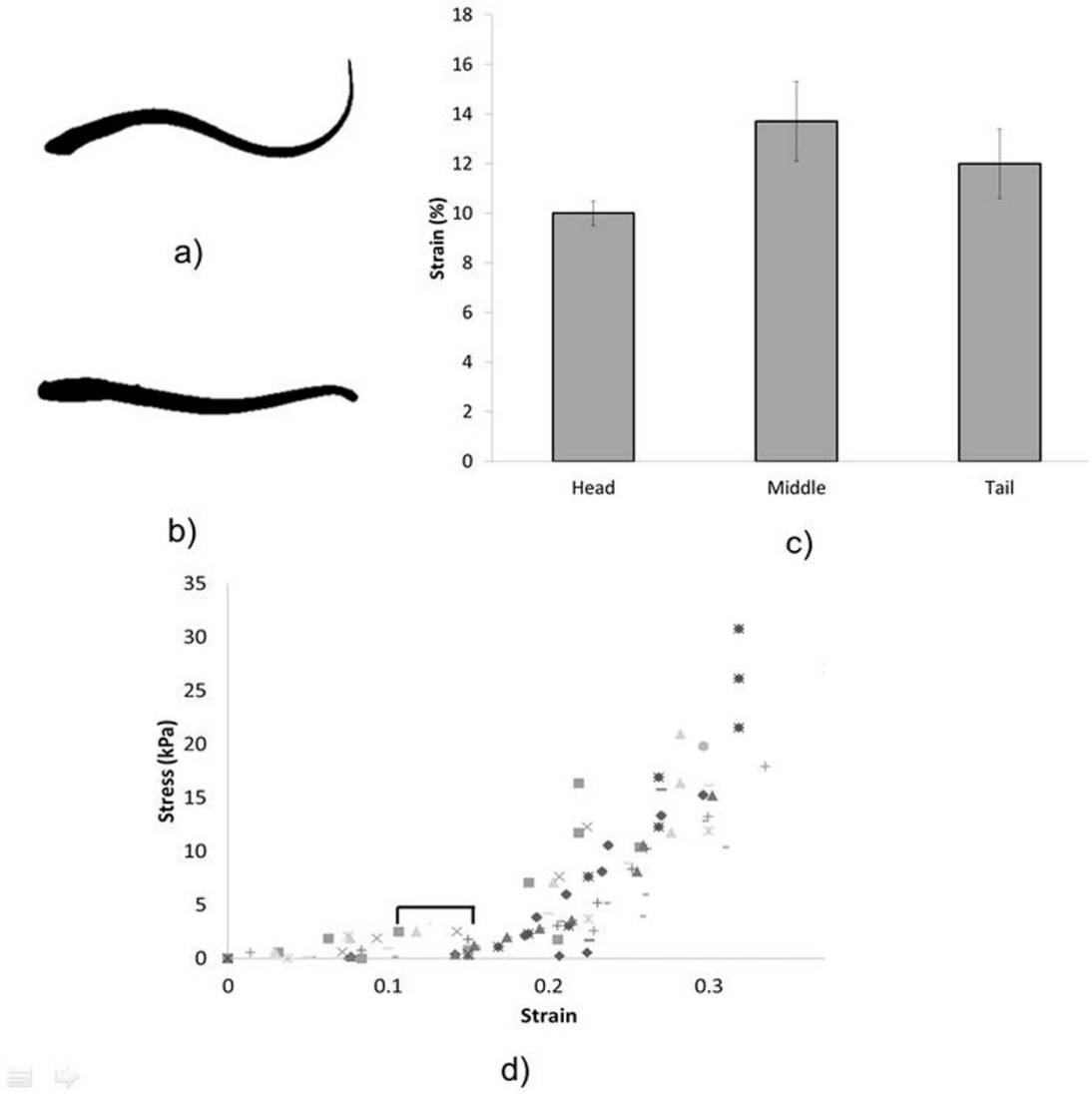


Figure 3-5. Mechanical properties of spinal cord. a) Black profile of a lamprey micrograph during normal swimming, b) Black profile of a lamprey micrograph at rest, c) Physiological measurements of uniaxial strain in the swimming position in the Head, Middle and Tail, d) Stress vs. strain data for spinal cord measurements (each symbol corresponds to a different spinal cord, N=10); the region indicated by a bracket (0.1 – 0.15 strain) corresponds to the physiological strain region. Statistical analysis was performed using Student's t-test and factorial analysis of variance (ANOVA) with a $p < 0.05$

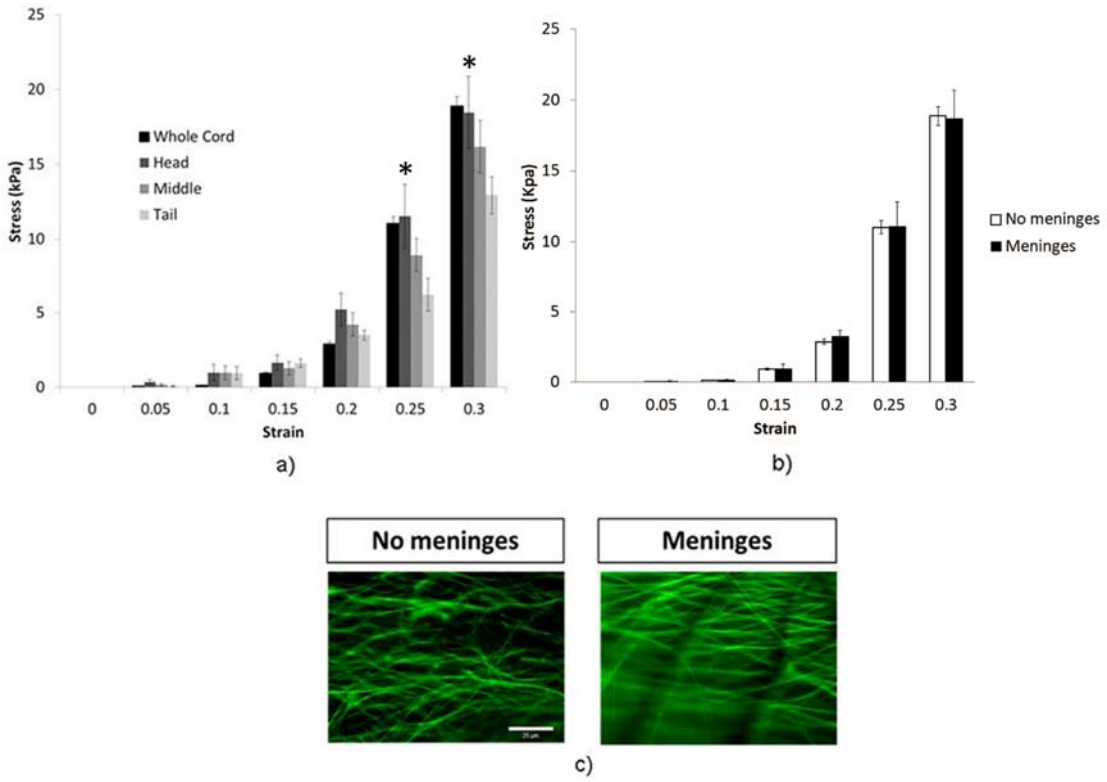


Figure 3-6. Stress-strain values of spinal cord with and without meninges and the different sections (Head, Middle and Tail). a) Average stress at different strain values for whole cord, Head, Middle and Tail sections, bars show standard deviation (N=5), b) Stress values at different strains for whole cords with and without meninges (N-5), c) Fluorescence staining of the spinal cord with (right) and without (left) meninges; showing that we were able to successfully remove the meninges. Asterisk in a) indicates statistical difference on the head sections vs middle and tail, statistical analysis was performed using Student's t-test and factorial analysis of variance (ANOVA) with a $p < 0.05$. Scale bar is 25 μm in all figures.

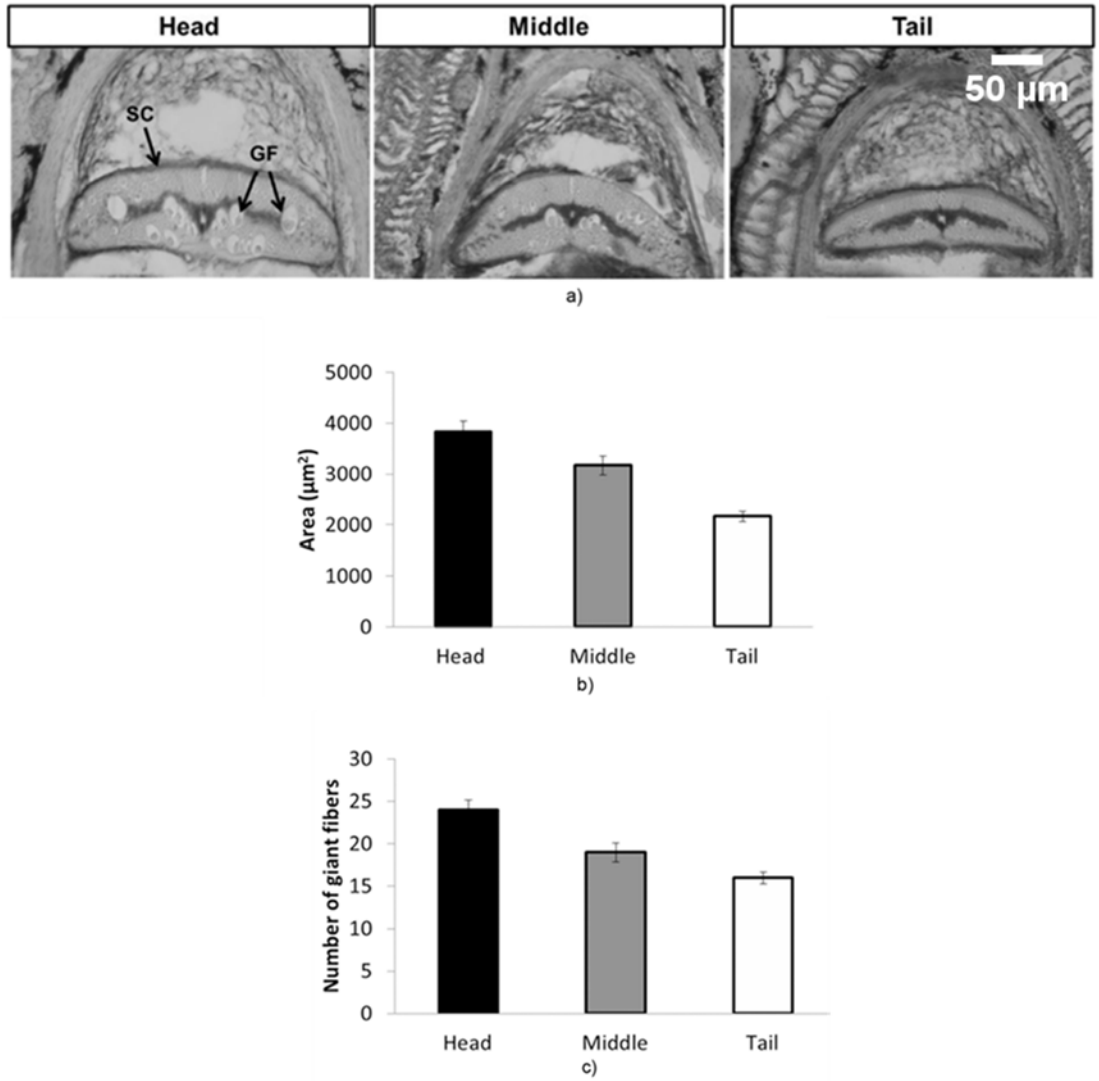
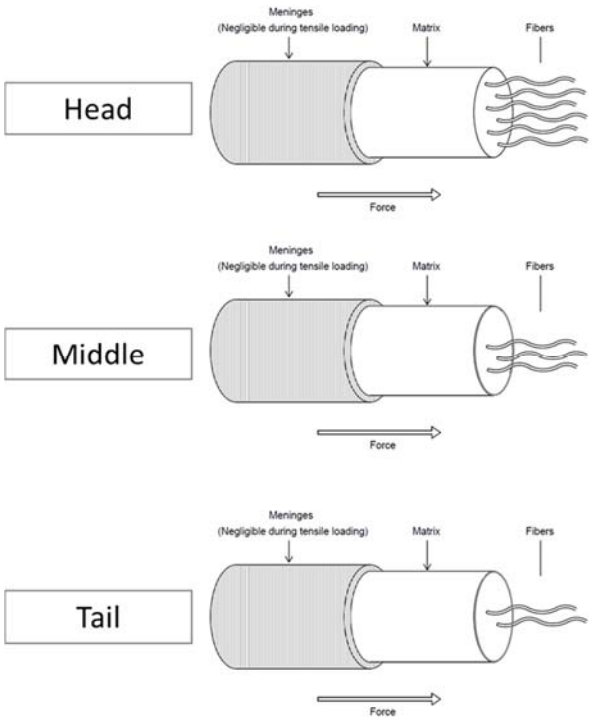


Figure 3-7. Histology of Head, Middle and Tail sections. a) Histological sections stained with toluidine blue, showing the differences from Head, Middle and Tail cross sections, arrows point at the large fibers (GF) and spinal cord (SC), b) Average areas of the spinal cord from Head, Middle and Tail, c) Average number of large fibers from Head, Middle and Tail. The large fibers were measured using toluidine blue stained cross sections, we considered large fibers to be those with a radius larger than 20 µm.



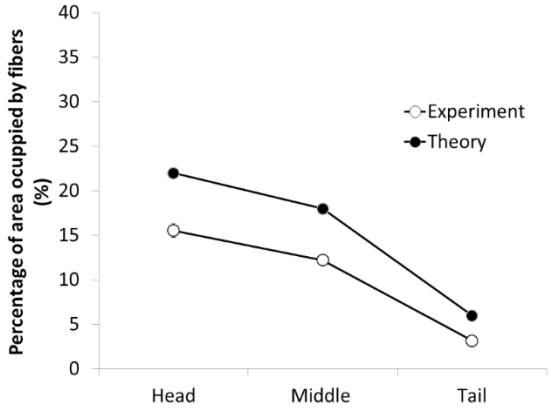


Figure 3-8. Percentage area occupied by the large fibers in the Head, Middle and Tail. Comparison of the values calculated by histological sections using toluidine blue (Experimental) and the values calculated using the composite material model (Theoretical), showing a similar trend from Head to Tail

3.2.2 Tensile loading of regenerated spinal cords

We measured the tensile loading response of regenerated spinal cords after 5 weeks. Unfortunately the spinal cords of maladaptive animals were fragile and we were unable to record any significant stress response during tensile loading. We were able to measure the spinal cords of animals regenerating in warm temperature (Figure 3-9). The regenerated spinal cords responded similar to control during physiological conditions (< 15 %), as the strain increased the stress response of regenerated cords was lower than that of uninjured cords (Figure 3-9 a). Then, we calculated the average number of fibers in the regenerated spinal cord and compared it to that of control. We found that the number of fibers responsible for tensile loading in regenerated cords is 20% less than the number of fibers in uninjured cords (Figure 3-9 b-c). These results further confirm our histological observations in which we concluded that regenerated spinal cords have fewer fibers compared to uninjured spinal cords.

3.2.3 Discussion

We found that the spinal cord has a pre-strain at physiological conditions, which is a very simple yet important finding. It indicates that after injury, the spinal cord will tend to recoil itself due to the already existent stress, which is an important mechanical effect to consider. If nerve function depends on strain [97, 98] then our results indicate that normal function is maintained for uniaxial strains between 10-15 %. In our search for the elements that affect longitudinal strain, we found no dependence on the meninges; in fact it can be seen (Figure 3-6) that they cross the spinal cord perpendicular to the direction of the applied force and the fibers. Thus, they are not

involved in uniaxial longitudinal strains but might be involved in lateral movements during compression or expansion.

Then, we explored the large fibers in the lamprey, which are parallel to the direction of the force and follow a distinctive, straight course throughout the spinal cord, with a size that makes them easily identifiable through histology [173]. Similar to the modulus at high strains, the number of fibers reduces from Head to Tail. This is due to the fact that they can narrow, loop, branch or shift position along the body [182]. As a result, the focus is placed on the fibers as the load bearing elements for high uniaxial longitudinal strains.

To simplify our study, we considered the spinal cord as a material made of two components, large fibers [173] and matrix [183] (Figure 3-2). In order to understand these elements under uniaxial longitudinal strains, we used a theoretical approach based on composite materials, via this model and our experimental data. We calculated the modulus of the matrix and fibers, as well as the approximate fraction of large fibers per section (Head, Middle and Tail).

According to the model, the modulus of the fibers is in the order of MPa, similar to the moduli found in humans, bovines and other animal spinal cords [114]. Also, the fiber modulus is much larger than the matrix modulus, a characteristic shown to be necessary for fiber support and reinforcement in composite materials [172]. We were

able to calculate the modulus of large fibers and matrix, which cannot be measured with conventional experimental methods.

Furthermore, using the uniaxial stress-strain curve, we calculated the percentage of fibers in each section (Head, Middle and Tail) with great accuracy compared to the results obtained with histology (Figure 3-7). This ability will be later used, to analyze regenerated spinal cords and calculate their respective modulus and percentage of fibers in the regenerated section.

The main limitation and advantage of the lamprey and the mathematical model is their simplicity compared to human spinal cords. Human spinal cord fiber tracts change directions as they descend from the cervical to sacral curve, which could affect the response to uniaxial tensile loading. Furthermore, the spinal cord contains more elements within the spinal cord such as veins and arteries and a more complex set of meninges [184]. Thus, human spinal cords will require the model and tensile analysis to be more complex. However, the principal concepts still apply, such as the pre-strain condition and the different uniaxial strain response due to the number of fibers.

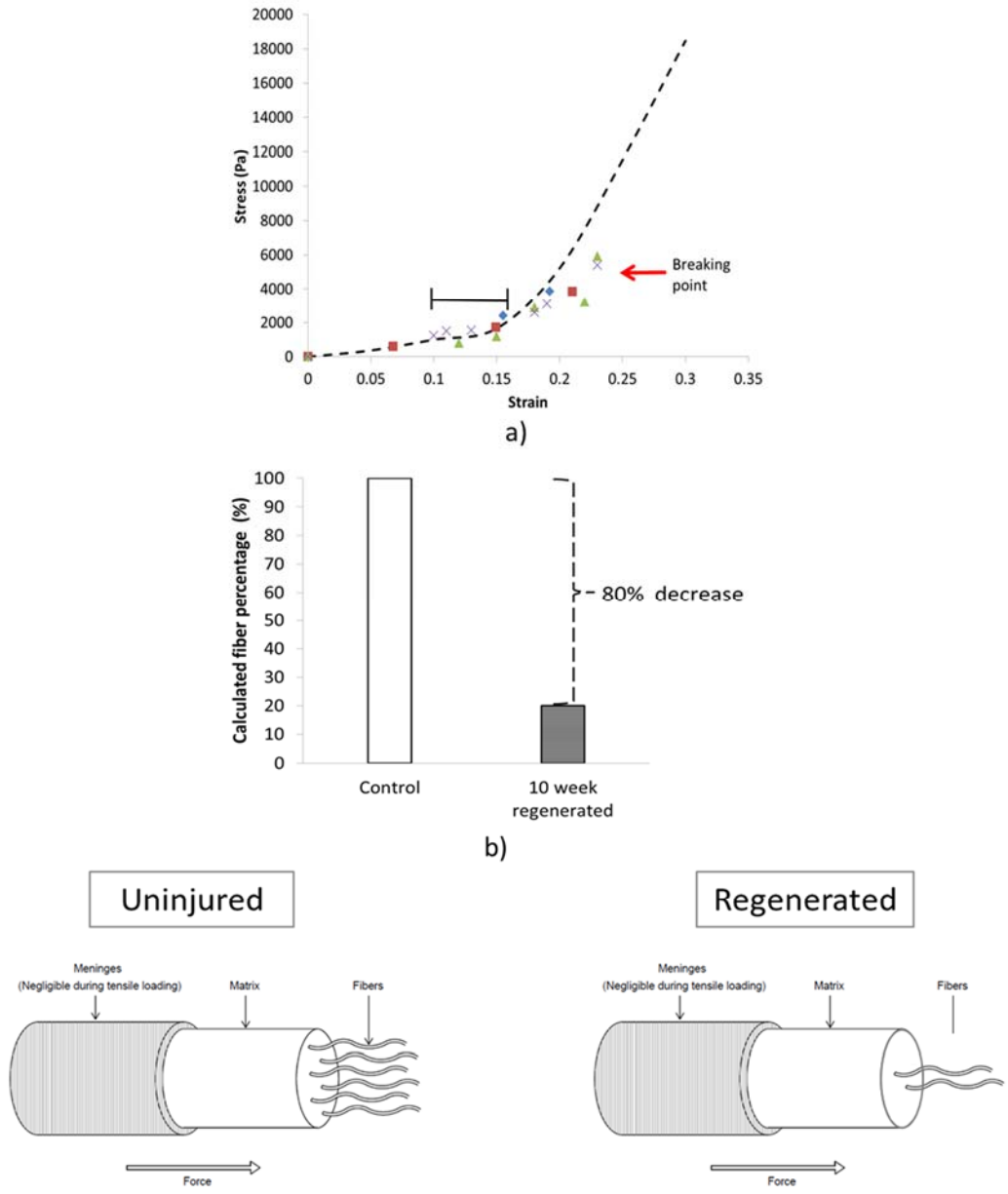


Figure 3-9. Mechanical properties of the regenerated spinal cord. a) Tensile loading measurements of regenerated spinal cords. Dotted line shows the average response of an uninjured spinal cord and colored symbols represent different regenerated spinal cords in warm temperature. b) Average number of fibers in a regenerated vs. uninjured spinal cord, calculated using the composite-material model. c) Schematic of results.

3.3 Biomechanics and imaging of the notochord before and after injury

3.3.1 Biomechanics of the notochord before and after injury

We measured the response of the notochord to uniaxial tensile loading by using the same set-up as the one used for the spinal cord. We measured the tensile response of uninjured notochords and notochords from animals regenerating in warm and cold temperatures for 5 and 10 weeks. Control notochords had a tensile modulus at low strains (< 20%) approximately five times larger than that of the spinal cord (60 kPa) (Figure 3-10 a). At higher strains the modulus was only 3 times larger than that of control spinal cords (150 kPa) (Figure 3-10 b).

At low strains the modulus of the notochord remained the same before and after injury (Figure 3-10 a). In warm temperature and cold temperature the tensile modulus was the same after 5 and 10 weeks of regeneration. At higher strains, the tensile modulus of animals in cold temperature at 5 weeks was the only one that differ from the rest (warm and control).

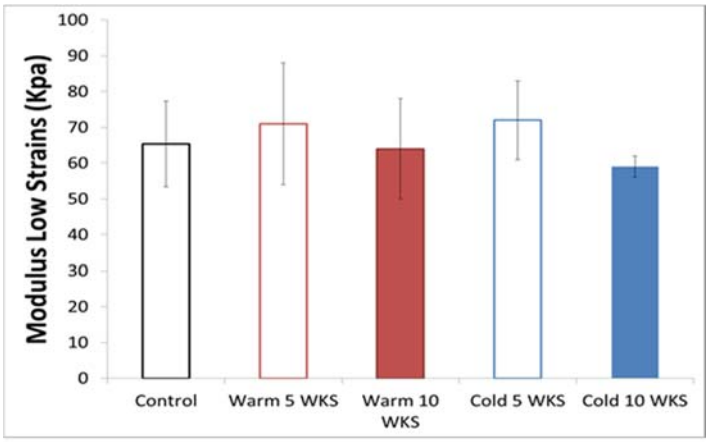
In cold temperatures, the notochord had a higher tensile modulus after 5 weeks (200 kPa vs. 150 kPa, Figure 3-10 b), but after 10 weeks of regeneration the tensile modulus was the same as that of warm and uninjured animals. In order to find what structural changes are occurring on the notochord after injury we decided to use X-ray phase contrast imaging on live animals.

3.3.2 XPC imaging of the notochord before and after injury

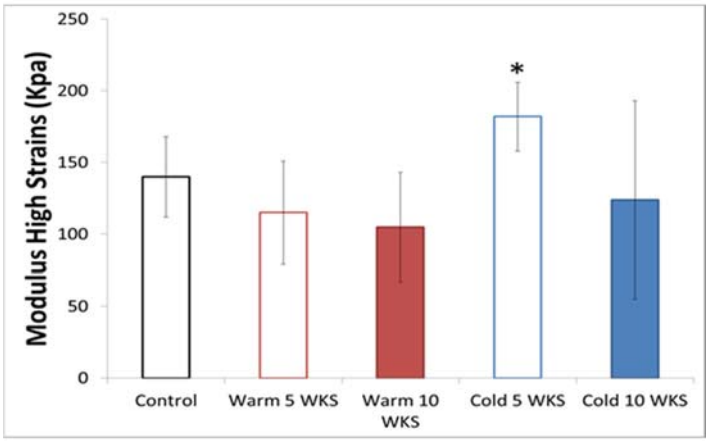
Using X-ray imaging on live animals without any contrast agent; due to their mineralized nature we could clearly observe the oolites concentrated in the head of the animal (Figure 3-11 c). Then, we moved to the notochord (Figure 3-11 d-e), using XPC we observed clear vertical seams that run around the width of the notochord (Figure 3-11 d). These segment seams are clearly a feature of the notochord (Figure 3-11 e) and appear to exist along the entire length of the animal. We analyzed the distance between segment seams in 4 live animals using XPC (Figure 3-12 a). The variation between segment lengths was around 20% but the overall length of the average segment was statistically similar between animals. These results were confirmed in isolated notochords, and suggest that no segment is identical to the next one.

Then, we imaged the effects of a spinal cord injury on the notochord. We carefully injured the spinal cord of animals and placed them back into the XPC approximately 5 minutes after injury. This amount of time was the minimum possible between transecting the spinal cord and placing the animal back into the beam. Once the animal was placed back, we observed striking and unexpected structural changes on the notochord (Figure 3-13). Although the injury was at the spinal cord, the notochord seemed to have changed its structure (Figure 3-13, right). The segment seams appeared to have reduced the distance between them, the upper border of the notochord seemed to be damaged and the lower border was bent towards the dorsal plane. These almost immediate changes indicated that the structure of the notochord was being affected by the spinal cord injury itself.

Next, we analyzed the changes in the structure of regenerated notochords in warm and cold temperature 5 weeks after injury (Figure 3-14). We found that the structure of animals in warm temperature resembled that of uninjured animals with straight upper and lower collagen borders. On the contrary, the notochord of animals in cold temperature was different. The upper border of the notochord was bent with a morphology that appeared to be a set of waves, the lower border was normal in most cases. Thus, we can conclude that the structure and mechanical properties of animals regenerating in maladaptive conditions (cold temperature) failed to recover the normal structure of uninjured animals. Animals regenerating in warm temperatures seemed to have a more permissive environment, since the notochord has recovered similar structural and mechanical properties of uninjured animals.



a)



b)

Figure 3-10. Mechanical properties of the notochord. a) Tensile modulus of uninjured and regenerated notochords after 5 and 10 weeks at low strains (<20%). b) Tensile modulus of uninjured and regenerated notochords after 5 and 10 weeks at high strains (<20%). Statistical analysis was performed using Student's t-test and factorial analysis of variance (ANOVA) with a $p < 0.05$.

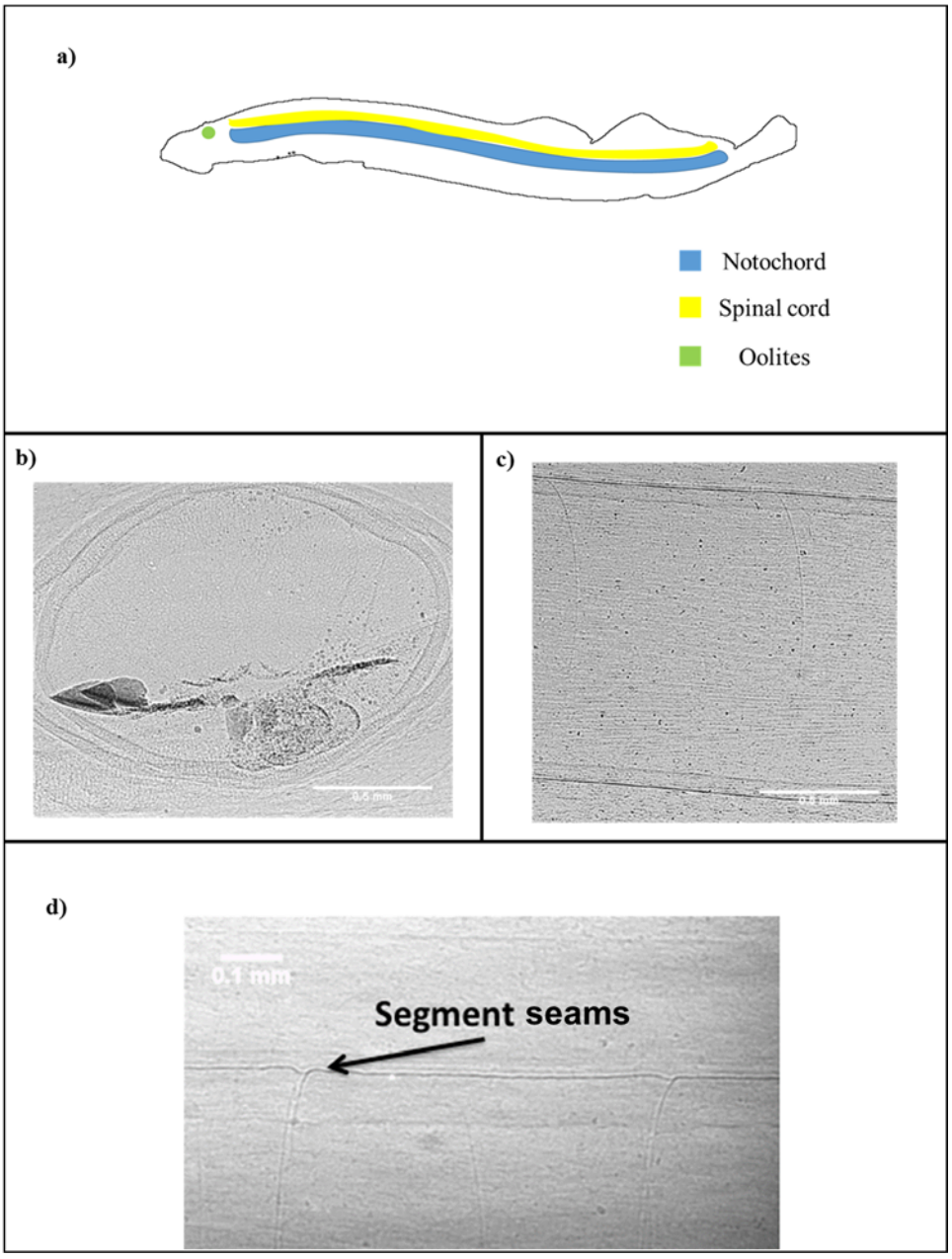


Figure 3-11. Live X-ray phase contrast imaging. a) Lamprey anatomy: Yellow-spinal cord, Blue-Notochord and Green-Oolites, b) X-ray absorption imaging of the Oolites, c) XPC imaging of the notochord with its boundary and d) Close-up on the segment seams found within the boundary layers. Scale bar in b) and c) is 0.5 mm and d) 0.1 mm.

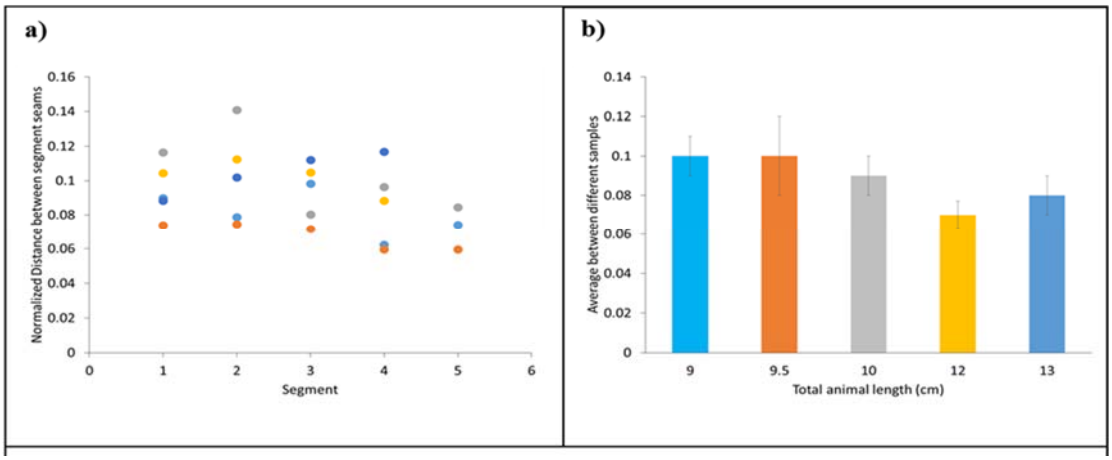


Figure 3-12. Distance between segment seams. Measurements were taken for 4 animals of the Petromyzon marinus species using live XPC, each color represents a different animal.

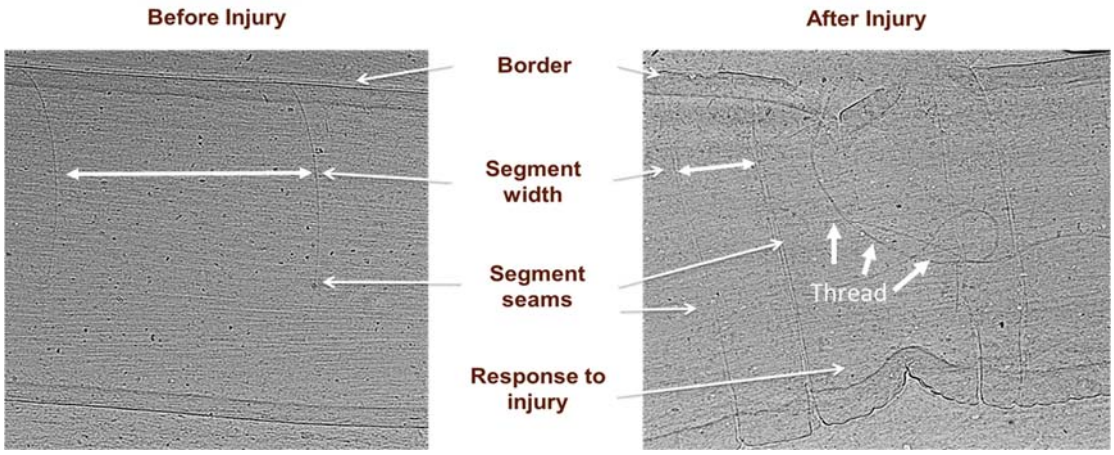


Figure 3-13. XPC immediately after SCI. Left, XPC of the notochord before injury. Right, XPC of the same notochord immediately after injury. A thread was placed at the injury site so that we could identify the exact location of the injury in the X-ray image, see arrow pointing at thread in right image.

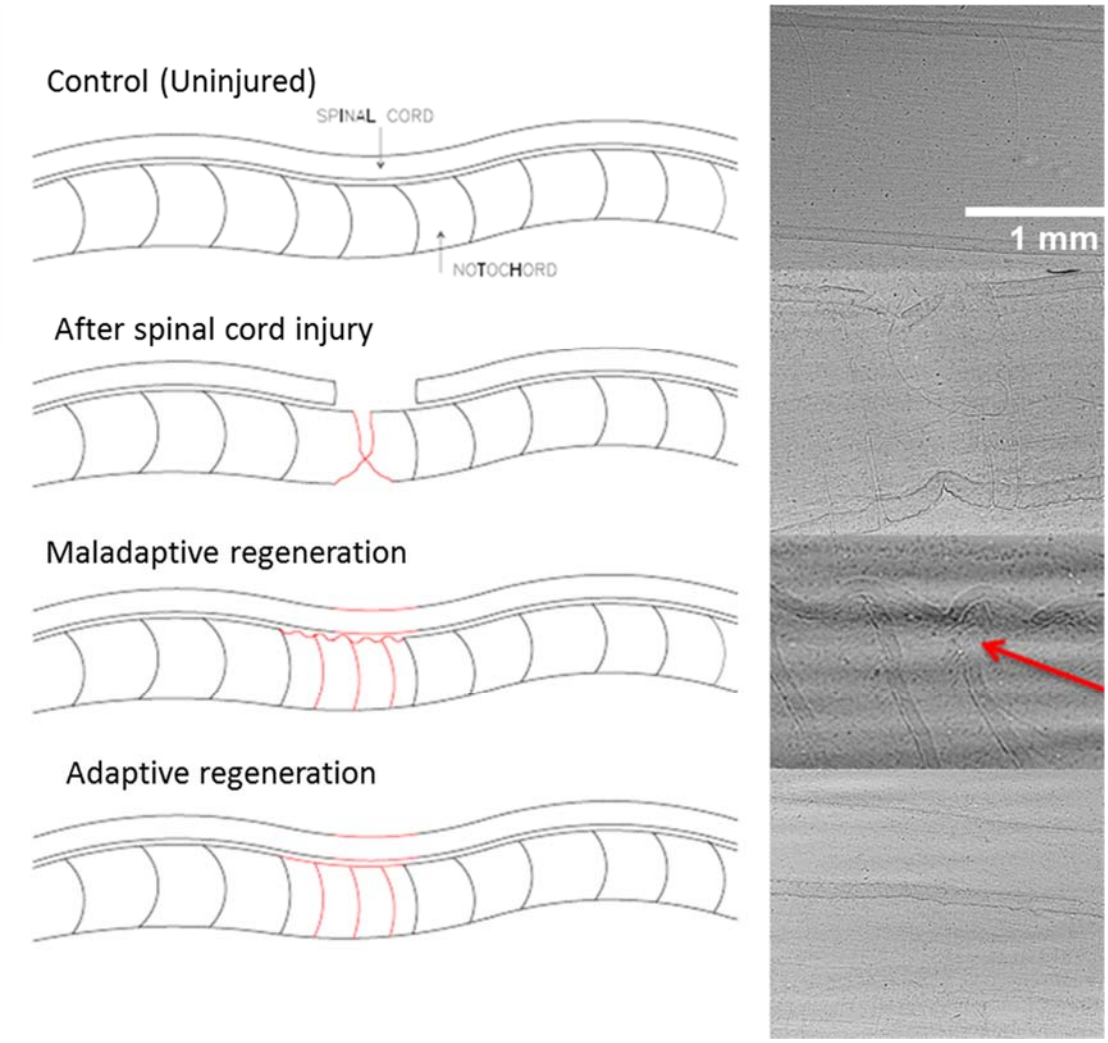


Figure 3-14. XPC of the notochord after regeneration. Right, XPC images of the notochord before injury, immediately after injury and 5 weeks after regeneration in cold and warm temperature. Left, schematic of the notochord according to XPC images. Scale bar is 1 mm in all figures.

3.3.3 Polarized imaging of the notochord

To confirm these results, we examined the lamprey notochord sheath under a light microscope. The isolated notochord appears to be clear and continuous (Figure 3-15 a), but when force is applied, the notochord sheath can be pulled apart into a set of rings of collagen (Figure 3-15 d). These collagen rings confirm the existence of a segmented rather than a continuous sheath structure.

Polarized microscopy, combined with histology, revealed more features of the collagen sheath (Figure 3-15 a). When stained with toluidine blue (Figure 3-15 c, d) we observed the fibers that compose the sheath. The fibers are aligned almost perpendicular to the long axis of the animal. Van Gieson's stain for elastic fibers revealed the segment seams along the notochord (Figure 3-15 e, f).

These seams are periodic along the length of the body and are vertical to the collagen fibers (Figure 3-15 e). Higher magnification shows that the seams are created by the absence of collagen (Figure 3-15 f). It is unknown what tissue is connected to the seams inside the body, information that may provide evidence regarding what additional role the segments play in the mechanics of the intact animal.

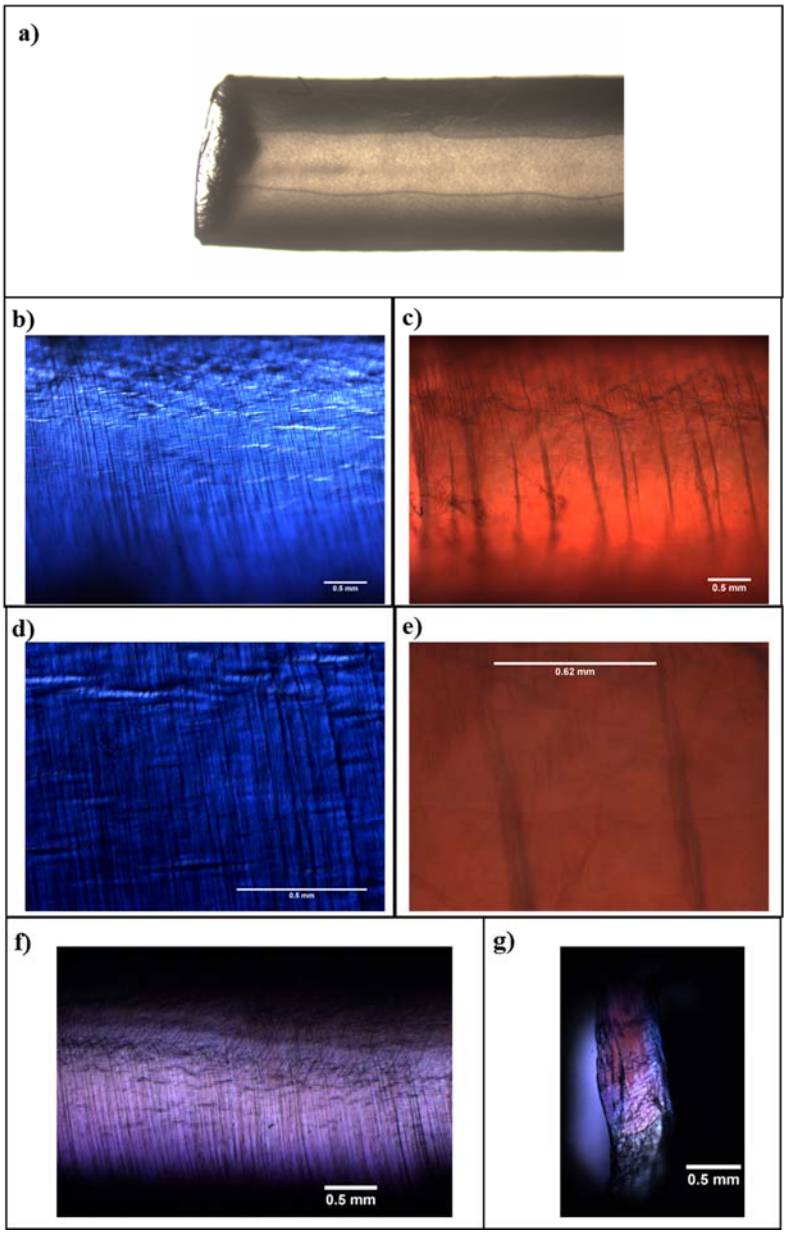


Figure 3-15. In vitro polarized microscopy. Further evidence of segmentation using histology and polarized microscopy from an isolated intact notochord. a) Bright field microscopy of the notochord, showing no segments. b, d, f and g) Notochord stained with toluidine blue, showing fiber orientation. c) and e) Notochord stained with Van Gieson's stain, showing the segmentation of the notochord g) Collagen ring mechanically removed from the notochord f). Scale bar is 0.5 mm in all figures.

3.3.4 Discussion

The reasons to have a segmented sheath remain to be studied; but it is safe to assume that it is linked to other anatomical units that are similarly segmented. In the lamprey, these units include the dorsal nerves, muscles and neural arches [185], all of which are involved in locomotion. In fact, it's well known that the notochord plays an important mechanical role during animal locomotion [136, 140-142]. The notochord sheath is strong but flexible, providing mechanical support that can resist high hydrostatic pressures, while allowing the animal to bend easily in different directions. The lamprey segmented notochord sheath (Figure 3-16) might be able to distribute the exerted forces individually in each segment, localizing the strains while maintaining a stable structure overall. If this is true, then having a segmented sheath is more efficient for lampreys than a continuous one.

Indeed, segmentation is an inherited property in many biological organisms. It allows for individual segments to specialize in response to a variety of needs, without having the need to create or replace a whole structure. It is an important evolutionary trait believed to have originated from a common ancestor of both arthropods and chordates [186]. Lampreys are extant early vertebrates and the fact that their notochord has marks of segmentation, might indicate that a segmented axial support was present in chordates before the existence of a cartilaginous or a bony skeleton. Segmentation in the notochord might not be unique. It may occur in other animals during the development of their vertebral column, and might exist even in animals that do not develop a cartilaginous or bony skeleton, but this remains to be demonstrated.

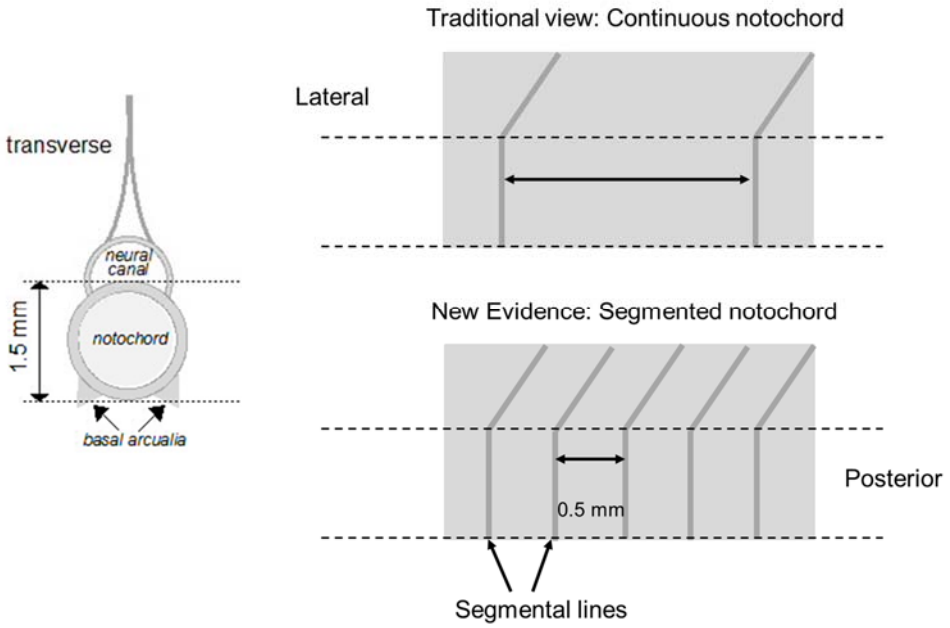


Figure 3-16 Schematic of the notochord with segments. Before our XPC experiments, the notochord was believed to be a straight continuous sheath of collagen (up) but we have demonstrated that the notochord is in fact segmented along the collagen sheath with an average distance of 0.5 mm (down).

3.4 Summary and conclusions

3.4.1 Importance of physiological strain and the mechanical properties

We measured the mechanical properties of the lamprey spinal cord and sections (Head, Middle and Tail) using a custom made tensile loading apparatus. We found that the modulus of the spinal cord at low strains is in the order of 0.015 MPa and at high strains of 0.5 MPa. We found that the spinal cord physiological strains are within the linear region (10-15%), similar to rabbit peripheral nerves [187]. There was no difference on the mechanical properties of the cord after removal of the meninges.

At high strains (> 18%), stress values in the Head and Middle sections are greater than stress values at the Tail section. We used a theoretical model based on composite materials to calculate the mechanical properties as a function of fibers and matrix ratio, that helped us understand the differences at high strains. We found that a decrease in the area fraction of fibers in the Tail causes the lower stress response. Using this model, we obtained an individual 2.4 MPa modulus for the fibers and a 0.017 MPa modulus for the matrix.

Understanding the stress response of the spinal cord holds a great potential in tissue engineering, for example in the creation of scaffolds with a similar modulus to promote regeneration. It is also important to understand spinal cord strain as it can influence function and nerve pathology [97, 98]. As a result, strain could impact functionality after regeneration. We have shown that, remarkably, changes in stiffness and morphology are correlated, with behavioral outcome: these changes occur only in

those animals that are most likely to have maladaptive behavior. There may or may not be a cellular and molecular response to these mechanical changes that triggers a different behavior in the animal. These cellular, molecular and mechanical changes are likely to hold the key to better understand not only spinal cord regeneration but the function of the spinal cord and the surrounding tissue.

3.4.2 Evidence of a segmented notochord, evolutionary and mechanical implications

The phylum of chordates and their subphylum of vertebrates are characterized by the presence of a notochord at some point during their life. During development, the notochord functions as a scaffold believed to pattern surrounding tissues, such as the spinal cord. It is also a hydraulic skeleton involved in locomotion that serves as the axial support. In vertebrates with backbone, such as humans, the notochord develops at the embryonic stage; remnants can be found in the intervertebral discs.

Lampreys are one of the few extant basal vertebrates that keep their notochord throughout their life. In research, lampreys are used to study neuronal circuits, animal locomotion and spinal cord regeneration. The notochord could play a role as a scaffold and axial support, and could prove important in studies of behavior and regeneration. However, classical histological techniques are limited to *in vitro* studies that can miss important physiological information. A better understanding of the structural and functional role of the notochord is more likely through the use of *in vivo* techniques.

In summary, evidence of a segmented notochord sheath was discovered in the living larval lamprey with XPC (Figure 3-16), and was confirmed with polarized microscopy and by mechanically removing collagen rings. The use of different methods supports our hypothesis that the notochord sheath of lampreys *(Petromyzon marinus*) is in fact, segmented along the body

Chapter 4: Enhancing spinal cord regeneration by clot removal: From maladaptive to adaptive recovery.

4.1 Experimental Methods and Rationale

4.1.1 Rationale

Spinal cord injury (SCI) is a physical trauma that can result in paralysis and even death. In humans, there exists no treatment to promote regeneration and even animals that are used for regeneration studies do not always recover functionally. Lampreys can recover head-to-tail motor coordination only in warm temperatures (23^0C), in cold/native temperatures (10^0C) their coordination is maladaptive. In our effort to understand maladaptive regeneration, we found evidence that, similarly to humans, lampreys in cold temperatures (but not in warm) form a clot at the injury site that might block/inhibit their regenerative capabilities.

We explored the hypothesis that removing the blood clot from animals in cold temperature will enhance regeneration and improve recovery. We created two groups of lampreys with spinal cord injuries at 10^0C. One group had the blood clot removed at 1 week using Gel foam®, while the other group was left without clot removal. Both groups were allowed to regenerate for a total of 3 weeks, after which swimming coordination was recorded. Those animals with the blood clot removed showed an improved coordination on their movements, while those without removal remained

maladaptive. These results supports the hypothesis that removing the physical blockade in spinal cord regeneration can enhance recovery.

4.1.2 Blood clot removal surgery

Larvae lampreys *(Petromyzon Marinus)* were anesthetized with 100 mg/ml MS-222 (Tricaine MS-222, Argent Labs, Redmond, WA). The animal was placed dorsal side up and the musculature opened with an incision at mid-body using a surgical blade. The spinal cord was exposed using a pair of tweezers and completely transected using a surgical blade, carefully avoiding any other tissue. Animals were divided into 3 groups and placed in temperature controlled rooms (Table 4-1). The animals were fed yeast once a week and their temperature and general health were checked daily. Animal maintenance and surgical procedures were approved by the University of Maryland's Institutional Animal Care and Use Committee, IACUC.

An hour after the spinal transection, 100 µL of a solution (1 x 10^8 beads/mL) of 1 µm polystyrene read fluorescence beads (Life Technologies, Gaithersburg, MD) were then injected in the blood clot using a 32G syringe with a 0.1 mm internal diameter (Sigma-Aldrich, St. Louis, MO). The lamprey then was returned to the ice bath for about 15 min before being returned to the aquarium (Table 4-1). Fluorescence images of the injury site were taken from live animals every 48 hours using a SteREO Discovery V20 Zeiss microscope with motorized 20x zoom.

For removal experiments, we injured 10 animals at mid-body and placed them in cold temperature (10 °C) for 1 week. Then, animals were taken from the aquarium, anesthetized and placed in an ice bath for 20 minutes. Using a surgical blade, we carefully opened the top of the injury site and removed the blood clot with a piece of GelFoam® dressing (Pfeizer Inc., New York, NY) without touching the spinal cord. Animals were left to regenerate for an additional 2 weeks and then their coordination was measured.

Condition	Coordination Analysis	Fluorescence and Optical Imaging	Time points
Warm temperature	10	5	0-3 weeks
Cold temperature without removal	10	5	0-3 weeks
Cold temperature with blood removal	10	5	1-3 weeks

Table 4-1. Animal groups.

4.1.3 Time frequency analysis of head-to-tail coordination

In order to analyze the recovery after injury, we measured the animal's head-to-tail coordination. Uninjured Lampreys oscillate their body (head, mid-body and tail) in a coordinated fashion (each traveling wave oscillates at the same frequency). We designed an experiment where the reference system was static by holding the tip of the animal's tail with a surgical thread. First, we anesthetized the animal, then using a 7-0 surgical suture with an elastic thread (Ethicon, Somerville, NJ) we pierced through the tail (0.5 cm before the end of the animal). The animal was left to recover and hanged vertically in a modified aquarium tank.

The tank was specially designed to allow only 2-directional movement of lampreys. Once the animal was awake, we recorded the motion using a digital video camera. We transferred the video to ImageJ (NIH, Bethesda, MD) and obtained the coordinates of the head, middle and tail sections as a function of time for at least 20 cycles of oscillatory movement. Then, we used Igor (Wavemetrics, Lake Oswego, OR) to do a fast Fourier transform and calculated the characteristic frequency for each section. Because the injuries were done at mid-body, we focused on the coordination between head and tail movements and calculated the difference between their characteristic frequencies. The closer the difference is to zero the better the recovery, and vice versa. Lastly, we performed a spectrum analysis using Igor in order to demonstrate the different frequency spectrograms between the head and tail of animals in cold temperature with and without clot removal.

4.2 Regeneration after blood clot removal

4.2.1 Blood clot dynamics

Once the fluorescence beads were injected into the blood clot of the transected lamprey (Figure 4-1 a), the animals were anesthetized and placed dorsal side up on the microscope. We obtained a bright field and a fluorescence image for each animal at the injury site (Figure 4-1 d-f). Then, we followed the overall displacement of beads over time by measuring the changes in the length of the bead distribution (Figure 4-1 g).

The procedure was repeated for animals recovering in warm temperature without removal and cold temperature (with and without removal of blood clot) (Figure 4-2). We observed that the bead distribution of animals in cold temperature without clot removal did not change over the course of 2 weeks (Figure 4-2 a, left column). On the contrary, the bead distribution in warm temperature changed in a manner which suggested to be an active directional transport (Figure 4-2 a, middle column). The dispersion of beads in cold temperature with clot removal further confirmed the success of the procedure (Figure 4-2 a, right column) demonstrating that the clot was effectively removed.

We measured the longitudinal displacement of beads over time in warm and cold temperatures (without removal, Figure 4-2 b). We found that the difference in bead displacement was more pronounced after the first week (Figure 4-2 b). In cold temperature, beads only displace 50% of their original length after 2 weeks. Meanwhile

at warm temperature the beads displace 250% of their original length, a difference of over 5 times compared to cold.

These results lead us to believe that removing the clot in cold temperature might enhance the regeneration of this animals to a more adaptive endpoint. Therefore, we removed the clots of animals in cold temperature 1 week after injury using GelFoam® (Figure 4-3). Three weeks after injury, we opened the injury site of animals without clot removal and still saw a great amount of blood (Figure 4-3 d). On the other hand, 3 weeks after injury (2 weeks after removal), the injury site of animals with clot removal appeared to be free of all blood clots and the spinal cord seemed to have regenerated (Figure 4-3 e). We then sought to analyze whether clot removal had improved or worsen the regeneration of animals in cold temperature.

4.2.2 Vertical static swimming and head to tail coordination

Uninjured animals swim by forming a traveling wave, where each section of the animal oscillates at the same frequency creating a coordinated movement. When injured, this coordination stops and it must be recovered for their regeneration to be called “adaptive”. We used this fact to analyze the effects of clot removal on lampreys. Our vertical static swimming set-up retained the static system of reference and restricted the movement of the animals to a 2-dimensional plane which improved the accuracy of our measurements (Figure 4-4).

Control animals have the same characteristic frequency at different sections of the animal (head, middle and tail, Figure 4-4). Animals left to recover in cold temperature from injuries at mid body lost much of their coordination and had a different frequency in each section (Figure 4-5 left). However, when the clot is removed, animals left to recover in cold temperature had a more similar frequency in each section (Figure 4-5 right).

Each animal recovers in a unique way, we demonstrated this by plotting the difference between the characteristic frequencies at the head vs. tail (Figure 4-6). An animal without injury has the same frequency in the head and tail (zero difference) thus, the greater the difference in frequencies the more maladaptive the recovery. For 6 animals with clot removal, 4 of them had an excellent recovery (difference below 0.5 Hz), one of them had a recovery above 0.5 but below 1.0 Hz and another had a more maladaptive recovery with a difference above 1.0 Hz. For 6 animals without clot removal, all of them had a difference above 0.5 Hz. Two of them were below 1.0 Hz and 4 of them were above, reaching a difference of almost 3 Hz. Interestingly, no two animals had the same difference between frequencies and when plotted in the same graph, their recovery followed a monotonic relationship

To better understand the frequency changes over time we calculated the spectrogram of head and tail movements for different animals. Control animals have a similar spectrogram in the head and the tail (Figure 4-7 left). The changes in the spectrum of frequencies at the head and the tail are coordinated; in other words, at the

same point in time, both the head and tail have a similar frequency spectrum. By contrast, animals that have maladaptive recovery showed a difference in the spectrogram for head and tail movements (Figure 4-7 right). We applied this analysis to animals in cold temperature with and without clot removal (Figure 4-8). We can clearly observe how animals without clot removal have a maladaptive recovery (Figure 4-8 right) and those with clot removal have a more adaptive recovery (Figure 4-8 left) as indicated by their frequency spectrums. The difference between head and tail frequencies and spectrograms indicate that animals in cold temperature with blood clot removal have a more adaptive recovery than those without removal.

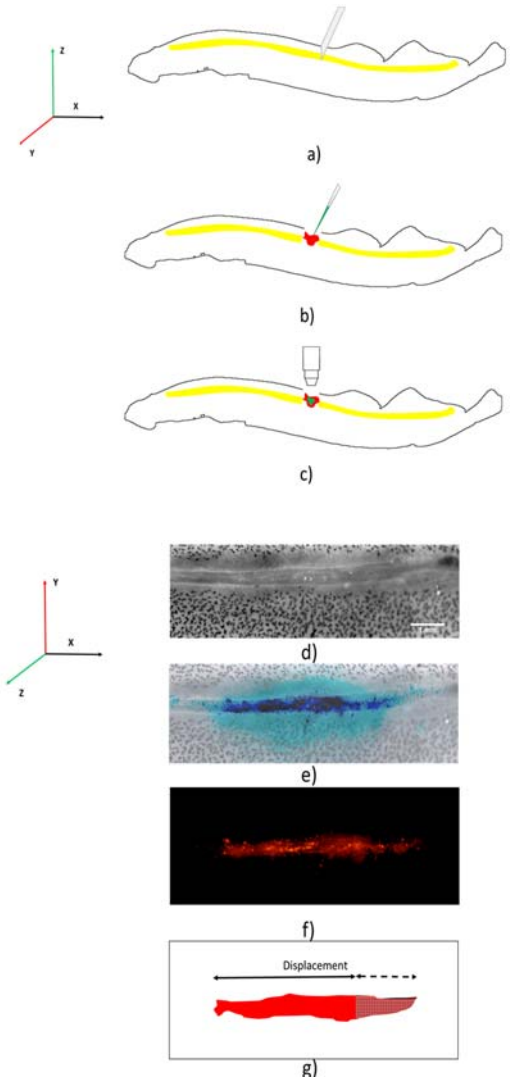


Figure 4-1. Fluorescence imaging of the injury site. a) Sketch of the lamprey, the spinal cord is shown in yellow. b) After injury beads are injected at the injury site, blood is shown in red and beads in green. c) Live lampreys are placed in a fluorescence microscope where imaging is done every 2 days. d) Bright field image of the dorsal part at the injury site. e) Superpose image of the fluorescent beads at the injury site. f) Fluorescence beads at the injury site. g) Diagram of the measurements for bead displacement. System of reference is shown in both left and right columns. Scale bar is 1mm in all figures.

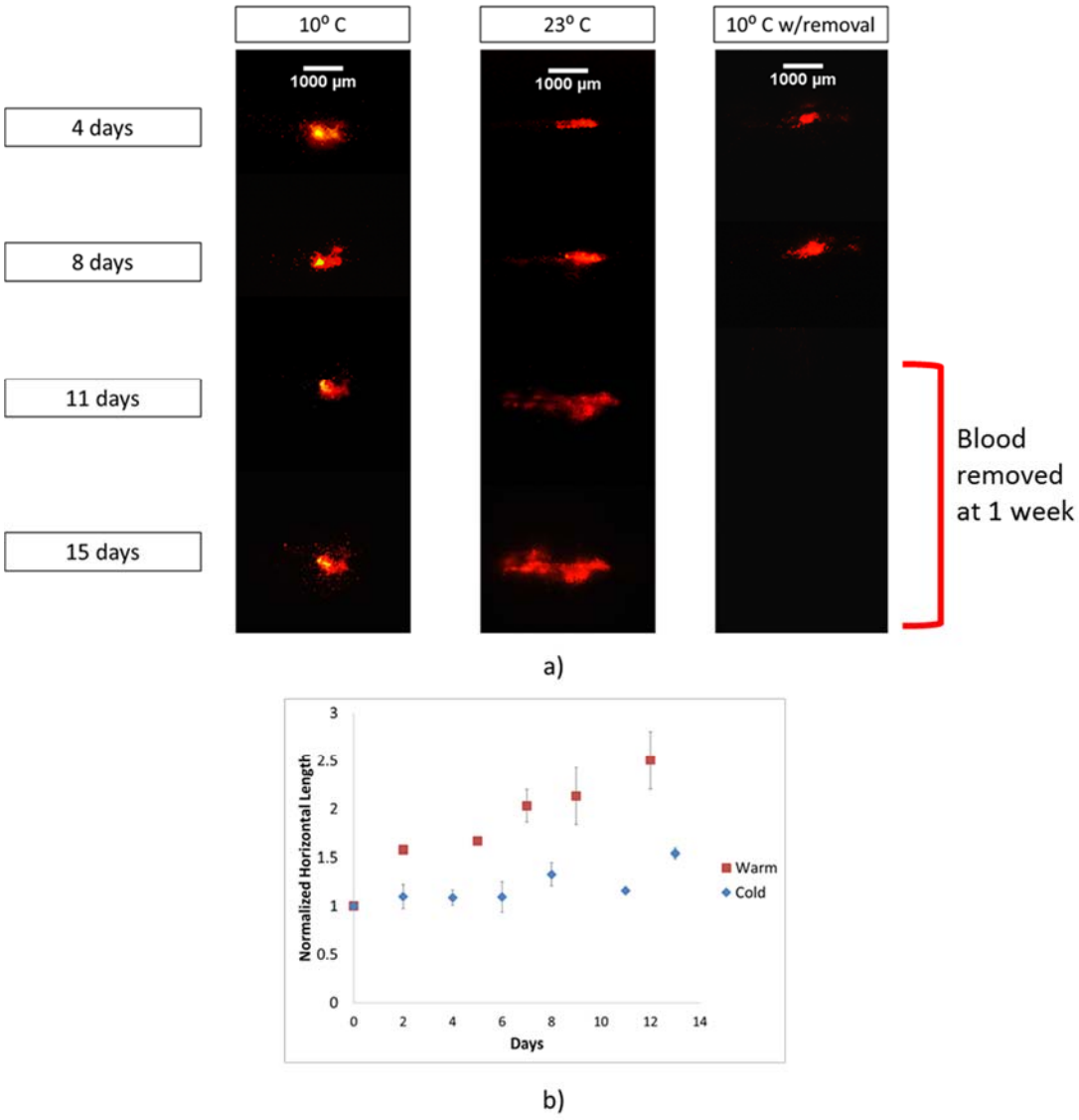


Figure 4-2. Fluorescent beads dynamics after spinal cord injury. a) Dynamics after 4, 8, 11 and 15 days after injury for animals recovering in warm and cold temperature (with and without removal). Blood was removed after 1 week, which is confirmed by the lack of fluorescently labeled beads on animals at 11 and 15 days. b) Displacement measurements 2 weeks after injury for animals recovering in warm and cold temperature. Scale bar is 1 mm in all figures.

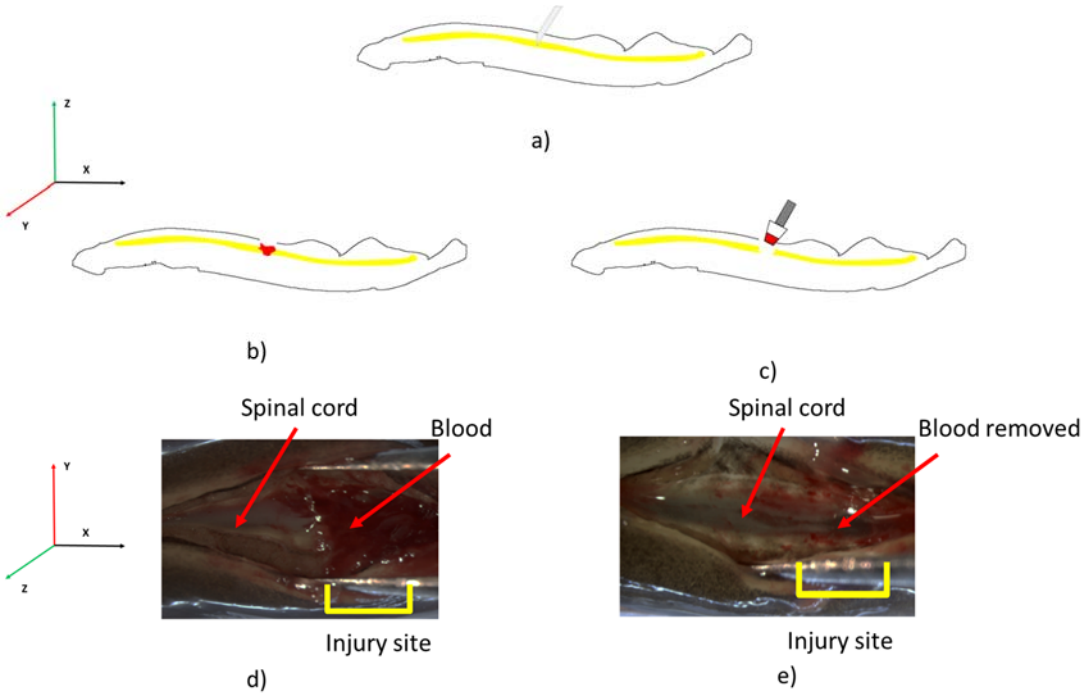


Figure 4-3. Blood clot removal. a-c) Diagram of blood clot removal, first we injured the lamprey and 1 week after injury removed the clot using GelFoam. d) Bright field image of a lamprey without clot removal 3 weeks after injury showing the blood at the injury site. e) Bright field image of a lamprey with clot removal showing the absence of blood at the injury site, 3 weeks after injury and 2 weeks after removal. The yellow arrows indicate the location of the injury site.

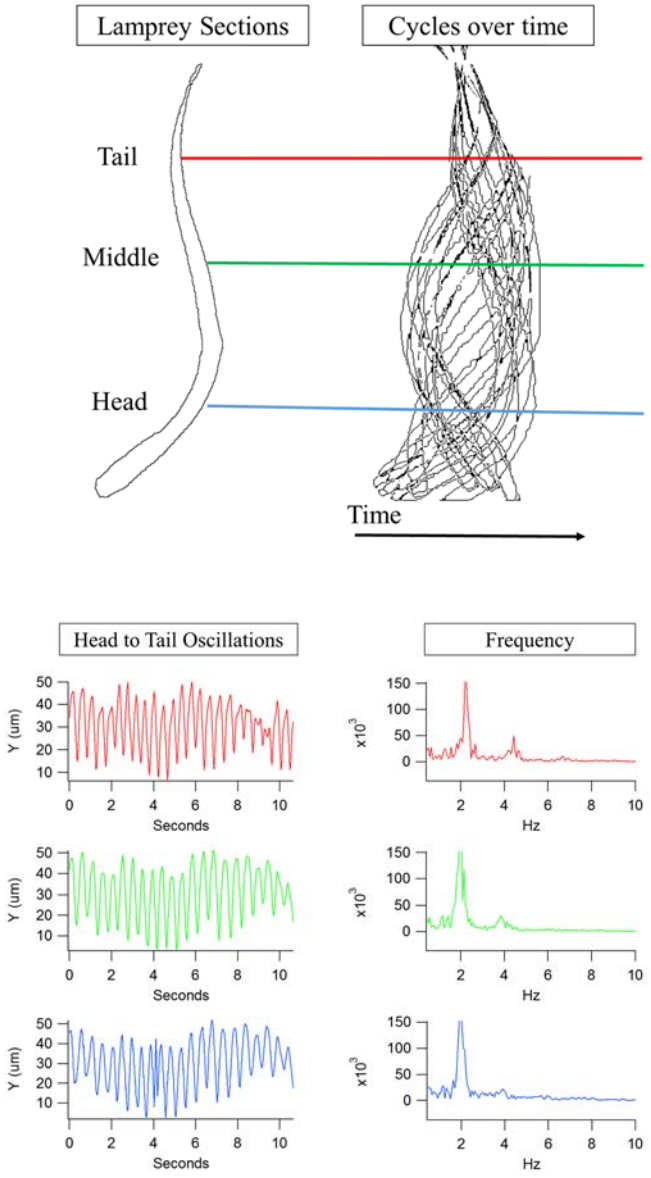


Figure 4-4. Head to tail coordination analysis. We used vertical static swimming to record the oscillations of the animal from head to tail. Then, we plotted the oscillations as a function of time we used a Fourier transform to obtain the characteristic frequency. In this figure we can observe an example of a control animal; all uninjured animals have the same frequency from head to tail.

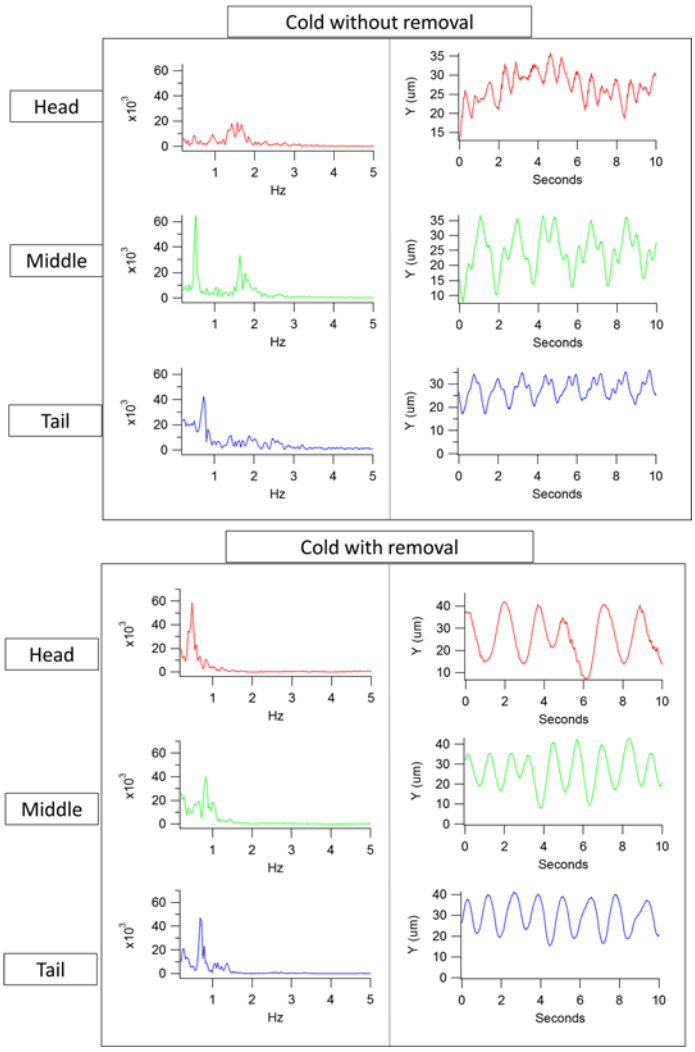


Figure 4-5. Frequency analysis of animals with and without removal. Left, cold animals without removal show maladaptive behavior, the frequency varies from head to tail. Right, cold animals with clot removal show a more adaptive behavior, the frequency is more similar from head to tail. The oscillations were obtained using animal recordings during vertical static swimming, each plot shows the oscillations of a specific segment of the lamprey body. The frequency plots were calculated from each sections oscillatory movement using a fast Fourier transform.

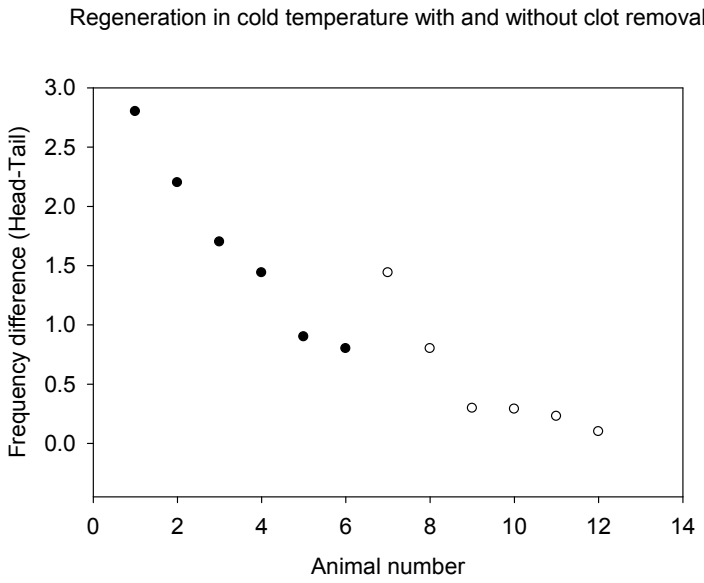


Figure 4-6. Frequency difference for animals with and without clot removal. Each animal is represented by a dot, black dots represent animals without clot removal and white dots represent animals with blood clot removal. The closer the frequency difference between the head and the tail is to zero, the more adaptive the recovery. We can observe that most of the animals with clot removal have a more adaptive endpoint compared to those without removal.

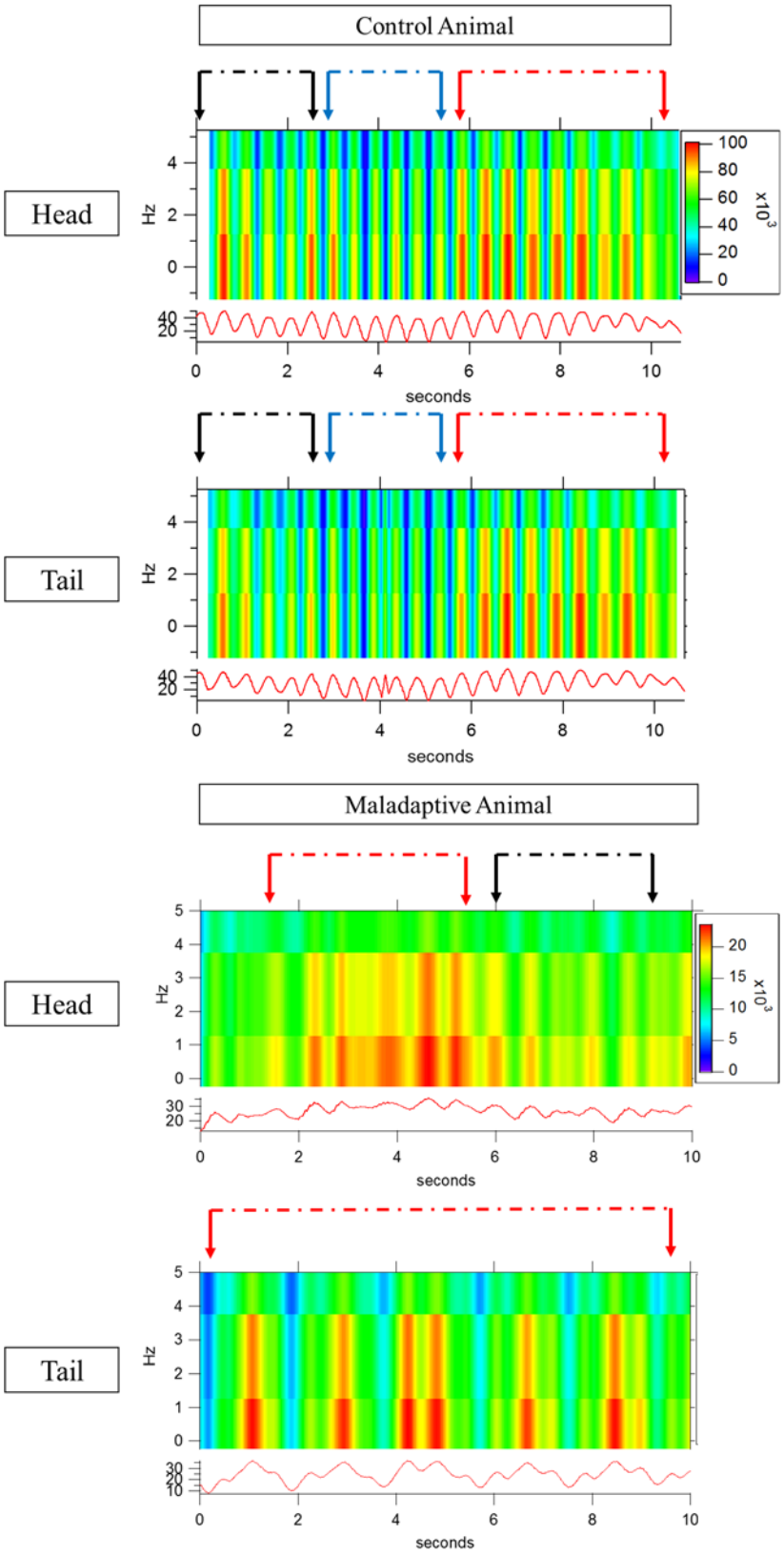

Control Animal
Head
Tail
Maladaptive Animal
Head
Tail
Hz
seconds
x10^3

Figure 4-7. Frequency spectrum analysis of animal locomotion. We calculated the frequency spectrum at the head and tail of animals before and after injury. Left, in the spectrogram of a control animal we can observe how the head and tail have similar spectrums as a function of time. Even when the frequency changes (black, blue and red dotted lines) both the head and tail maintain similar oscillations. Right, maladaptive animals have different spectrograms for the head and the tail, demonstrating the lack of coordination between their movements as a function of time.

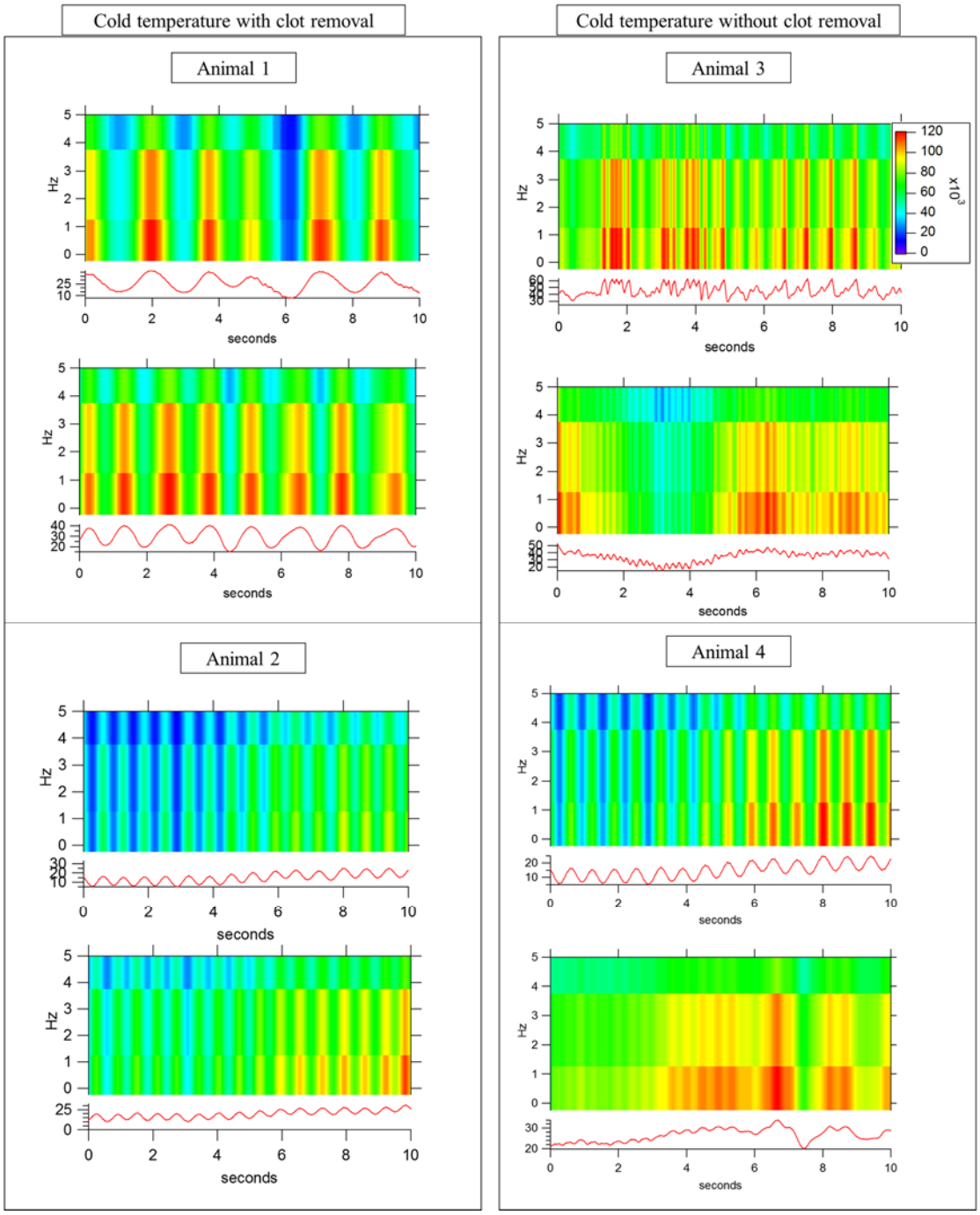

Cold temperature with clot removal
Cold temperature without clot removal
Animal 1
Animal 3
Animal 2
Animal 4
Hz
seconds
x10^3

Figure 4-8. Frequency spectrum analysis of animals with and without clot removal. In this figure we show the spectrogram of 4 animals, 2 with and 2 without blood clot removal. We can observe that for animal 1 and 2 which had clot removal, the spectrogram of the head and the tail are similar. Animals 3 and 4 which didn't had clot removal have different spectrograms from the head and the tail. These results demonstrate that the regeneration in cold temperature with clot removal has a more adaptive recovery than without removal.

4.4 Summary and conclusions

After spinal cord injury, it is widely accepted that the human response creates a non-permissive environment that physically blocks regeneration [15]. We used the dual regenerative capabilities of the lamprey (mostly adaptive at 23 °C and mostly maladaptive at 10 °C) to explore whether a similar obstruction was present in maladaptive animals. We used the transport of fluorescent beads at the injury site in warm and cold temperature as a way to observe the lamprey's ability to clean the injury site. We concluded that 1 week after injury, the beads were transported away from the injury in warm but not in cold temperature (Figure 4-2). This indicated that in cold temperature the lamprey ability to clear the injury site is hindered and might be responsible for the maladaptive behavior.

Second, we hypothesized that by removing the blood clot at the injury site of cold animals we could enhance their recovery to a more adaptive endpoint. After we removed the blood from the injury site, we quantified lamprey head to tail coordination by using a time frequency analysis (Figure 4-8). This allowed us to analyze the difference in locomotion of individual animals in cold temperature, with and without clot removal. We established a range of adaptive and maladaptive endpoints, from the animal with less coordination to the animal with a coordination similar to that of uninjured lampreys (Figure 4-4). In this range, lampreys with blood clot removed had a coordination more similar to uninjured lampreys and those without clot removal were mostly maladaptive.

In our group of lampreys after clot removal, we had 4 animals that recovered adaptive coordination and 2 that had a more maladaptive endpoint. Without clot removal, all of the animals had a maladaptive endpoint. The difference in head and tail coordination of those maladaptive animals varied linearly from 0.5 to 3 Hz. A similar linear trend of maladaptive behavior has been observed using electromyography on the muscle and spinal cord of lampreys in cold temperature. The factors that vary between individual animals which affect the coordination in such a linear fashion remains to be understood. Future work could investigate the role of the nerves, neurotransmitters and other molecules that could be involved in the varying maladaptive and adaptive endpoints. The non-invasive analysis using vertical static swimming and head-to-tail coordination can be of great use to determine the functionality of regeneration without harming the animal. This could allow researchers to use the same animal for other invasive procedures and correlate the level of coordination with the results from procedures such as histology and immunostaining for individual animals.

Understanding the degree of recovery is important because what in lampreys translates to lack of coordination, in humans could translate to respiratory, cardiovascular, motor and sensory malfunctions. Thus, we stress on the fact that nerve regeneration does not equal successful recovery and that there exists a range of recovery, even in lampreys in the same temperature, with or without clot removal. The existence of maladaptive regeneration has been clearly shown for the lamprey, but it doesn't necessarily mean it exists only in this animal model. In fact, we believe that

research done in other animal models demonstrates hints of maladaptive regeneration. For example, studies done in newts [56] and eel [54] report that not all animals recovered normal function after injury

Unfortunately, simply stopping coagulation cannot solve the problem of nerve regeneration. SCI is after all, an injury that without coagulation could cause internal hemorrhage, external excessive bleeding and death [188]. But the same process can also cause obstructions of blood flow and nerve regeneration that induce apoptosis and hinder behavioral recovery [189, 190]. Thus, SCI cannot be viewed as a simple static process, but a time dependent one in which is important to understand when to leave a clot and when to remove it.

Treatments after spinal cord injury have been found to be more efficient early after injury, after a few days or weeks [191, 192]. Unfortunately, there is not much information on spinal cord injury and/or regeneration during that period of time. Therefore, we sought to use fluorescent beads to track the transport of beads away from the injury site every 48 hours during a period of 2 weeks, for warm and cold temperature. This technique allowed us to measure the rate at which adaptive and maladaptive animals were able to clean the injury site from fluorescent beads. We were able to measure that the greatest difference between the displacement of beads in warm and cold temperature, occurs 1 week after injury. These results helped us hypothesized that regeneration in cold temperature could be enhanced by removing the blood clot 1 week after injury

We used fluorescence imaging to better understand the behavior at the injury site, which is further proof that the creation of technologies that can detect and monitor spinal cord injuries early after injury (in humans) is key to the development of repair strategies. It is also important to consider that one treatment might not cure all spinal cord injuries. In some cases a pharmacological agent could help recover locomotion and in others the removal of fluid might help recover a better neurological outcome. As the field of spinal cord regeneration advances, it remains to be seen what the combination of molecular therapies with surgical treatments could do to the neurological outcome after SCI.

Chapter 5: Summary and Future directions

5.1 General Summary

5.1.1 Spinal cord regeneration: Adaptive vs. Maladaptive vs. Clot removal

Overall, from the work generated by this thesis project we have learned some of the physical factors that differ between adaptive and maladaptive recovery. We believe that the results from this work are not restricted to lampreys and the general principles can be translated to other animals. The main principles learned from this work are the following:

Specific Aim 1: Adaptive vs. Maladaptive

1. The spinal cord **in warm temperature regenerates faster** than in cold temperature.
2. The difference in regeneration might be due to the **blood clot that is present in cold temperature** but not in warm.
3. **The meninges does not seem to be involved** in the regeneration of the spinal cord in both temperatures.

Specific Aim 2: Mechanics and structure

1. The **spinal cord in physiological conditions is under stress**, which causes it to recoil after injury.

2. After regeneration in **warm temperature**, tensile loading measurements indicate that the only **few of fibers have regenerated**, but enough to **recover the mechanical properties at physiological conditions**.
3. The notochord of lampreys is **segmented** and suffers mechanical damage after injury.
4. In **cold temperature**, the notochord **does not recover** the morphological features of uninjured notochords.
5. In **warm temperature**, the notochord **recovers the morphological and mechanical properties of uninjured notochords**.

Specific Aim 3: Clot removal and head to tail coordination

1. In cold temperature, animals are **unable to clear the injury** site of fluorescent beads.
2. After **manually removing the blood clot**, animals in cold temperature **recover to a more adaptive endpoint** (compared to animals without removal).
3. A time frequency analysis of head and tail coordination can be used to measure the different levels of recovery for each individual animal.

All of our results point at one single scenario: in cold temperature the spinal cord has an inhibitory environment that results in maladaptive recovery. The first event starts with the injury itself, a complete transection to the spinal cord cuts every fiber

and the release of mechanical tension (recoil) creates a larger gap between each nerve-end that must be regenerated in order to form connections. Furthermore, it appears that the damage is not independent to the spinal cord, as we have shown that the notochord right after spinal cord injury, shrinks and collapses at the injury site. These events are immediate after injury and independent of the temperature, chronologically they are the first physical factors that must be repaired in order to achieve regeneration.

Following this chronological order, the next physical factor was found to be the blood clot at the injury site. In this case, we have shown that animals in cold temperature are unable to displace the blood (and fluorescent beads) away from the injury site, which leaves a physical blockade between the nerve ends. This physical factor might be the most devastating, the spinal cord in cold temperature might still be able to regrow, but with a physical blockade in the way regeneration is inhibited.

The blood clot and the mechanical changes were observed within a 2 week time frame and regeneration in cold temperature might eventually be achieved after many more weeks. But it was our objective to understand the early events, and analyze the factors that can affect the behavioral outcome. As we have shown in our last aim, we believe that treatments done early after injury will have the most effect to promote a functional outcome, versus treatments that are done late after injury, when a complete glial scar has been formed and most neurons have suffered apoptosis. Removing the blood clot could be a much easier treatment than removing the glial scar, which contains a greater number and greater variety of cells.

Therefore, one of the most promising parts of this work, is the fact that completely removing the blood clot enhances the regeneration of maladaptive animals. This is important because it shows that the outcome of regeneration can be changed by removing the physical blockade. In this case, the lamprey is a simpler organism than humans and the surgical procedure to remove the blood clot was relatively easy. Human anatomy and physiology after injury is much more complex and understanding when and how to remove the physical blockade will be a bigger challenge. But our results indicate that searching for a way to physically remove this blockade might give us a greater reward than a challenge. The development of new technologies to diagnose spinal cord injuries and the advancement of surgical techniques will without doubt make the challenge of removing such physical blockades easier.

5.1.2 Blood coagulation and the immune response

It is clear that the blood clot is one of the main players that influences recovery after SCI. In warm temperature the blood clot disappears early after injury, but in cold temperature the blood clot blocks regeneration during the 2 week period that we covered in this study. Although the scope of this work was not to study the lamprey's immune system, we investigated what is the involvement of blood after SCI in the lamprey and other animal models.

A blood clot is formed via a natural process in which blood coagulates after injury to prevent excessive bleeding [193]. Lampreys formed a clot in response to the

intrusive injury performed in our experiments. But this response is not independent to lampreys, most animals (most importantly humans) will also create a clot to prevent bleeding, especially after an injury to a major tissue such as the spinal cord. Therefore, an interesting question is: how is the immune response in warm temperature different than in cold, and what are the changes that allow for the injury site to be clean of a blood clot in warm but not in cold temperature?

Such a contrast in the same animal model is impressive, mainly because only by changing 1 variable (temperature) we have remarkable differences in recovery. Similarly, by only removing the blood clot without the aid of any pharmacological agent we were able to change again the outcome of lampreys, this time from maladaptive to adaptive. The fact that removing the clot yielded a positive result does not indicate that this will happen with every treatment, thus the necessity to carefully analyze animal behavior and establish a range of recovery levels.

Human spinal cord injuries might present a more complicated case. Not only there is a more complex anatomy and physiology, but also the damage can vary greatly depending on type of injury. Thus, it is important to develop technologies that can detect and monitor the spinal cord injury over time. It is also important to consider that one treatment might not cure all spinal cord injuries. In some cases a pharmacological agent could help recover locomotion and in others the removal of fluid might help recover a better neurological outcome. As the field of spinal cord regeneration

advances, it remains to be seen what the combination of molecular therapies with surgical treatments could do to the neurological outcome after SCI.

5.2 Future Directions

5.2.1 Electrophysiology studies after clot removal

Although our time frequency analysis using videos of lampreys can measure the level of coordination between body segments, another tool that will give information about individual and groups of fibers is electrophysiology. Regenerated spinal cords could be used for electrophysiological experiments; by doing this researchers could obtain more information about fiber connectivity and the level of coordination between rostral and caudal fibers.

Regenerated spinal cords from warm and cold temperature, with and without removal could be placed in a cooled chamber (10 °C), super fused with lamprey saline, prepared as previously described [169]. Then, recording electrodes could be placed above the rostral and caudal sections with respect to the injury site (Figure 5-1). In order to obtain locomotor signal, fictive swimming could be induced with application of d-glutamate (0.25-0.50 mM), and the two burst nerve recordings digitalized and rectified using custom software. For analysis of functionality, bursting of muscle or spinal cord fibers could be examined for evidence of abnormal coordination between segments rostral and caudal to the lesion site (Figure 5-1).

Spinal cord function could be tested by looking at the coordinating system during fictive swimming in similar fashion as previously reported [169]. Electrophysiology has the disadvantage that the tissue will no longer be usable for other techniques such as histology and that the animal has to be sacrificed; but it has the advantage that it can record burst of individual or groups of fibers as well as muscle recordings. For experiments were the animals need to survive and structural measurements are needed, researchers could use the time frequency analysis presented in this thesis to correlate the functionality of animal locomotion.

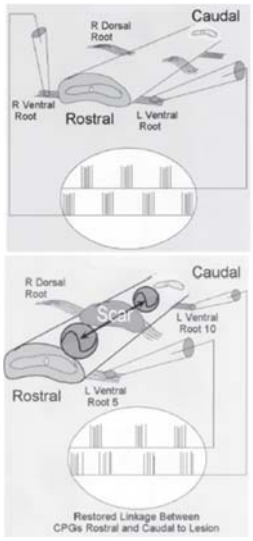


Figure 5-1. Electrophysiology on isolated spinal cords. Frequency burst can be measured in the rostral and caudal sections of spinal cords to analyze their level of coordination.

5.2.2 Molecular studies in adaptive and maladaptive animals

In this thesis we have carefully characterized the morphological and mechanical changes of lampreys after regeneration. However, there is also a need to gain an understanding of the changes at the molecular and cellular level. Thus, future directions should include the use of cellular (Table 5-1) and molecular techniques (Table 5-2, 5-3) to understand the differences between regeneration in cold and warm temperature, with and without clot removal.

Alternative experiments might include looking at specific proteoglycans and proteins in the scar, however due to time and number constrains we summarized some of the most important molecules in Table 5-2 and included a complete list of molecules in Table 5-3. These tables not only helps us with future directions, but can also be a help for potential pitfalls. For example, if GAG staining does not work or the expression of CSPGs is not specific, researchers might try to look at more specific proteoglycans. Also, if netrin-1 is not found in the spinal cord then researchers could try other molecules such as Nogo or MAG. Other experiments besides immunostaining can be done in the area of genetics and microbiology.

The expression of CPGs, myelin-associated proteins and neurotransmitters might be different for adaptive and maladaptive animals. Immunostaining methods of frozen and paraffin sections could be adapted from [56]. Stain for the GAG section of CSPGs, could give a general illustration of CSPG expression and localization and researchers could analyze the differences between adaptive and maladaptive animals.

Other experiments could include expression using antibodies for NG2, neurocan and phosphacan which are proteoglycans up regulated after stab injuries [194-196]. For the expression of myelin-associated molecules, researchers could choose Netrin-1 since the receptors have been found in the lamprey; however if nothing is found another option could be to use Nogo, MAG or OMgp antibodies.

For immunostaining in the lamprey, the specificity of the secondary antibodies should be verified by treating adjacent sections with secondary antibodies in the absence of primary antibodies. Since paraffin processing may affect GAG labeling, CSPGs must be analyzed in frozen sections. Expression patterns could be verified in whole-mount preparations of isolated spinal cords. In summary, future directions for cellular and molecular work could be divided in two set of experiments: Group 1) Cellular elements (axons, glial cells, blood vessels) and neurotransmitters, Group 2) CSPGs, myelin-associated proteins and adhesion molecules. In this section we have provided two tables that will help easily design this set of experiments in lampreys, although all of these expression experiments could be easily translated to other animal models, only in lampreys we could analyze the difference between adaptive and maladaptive behavior.

Glial Cells

Staining Method	Reagent	Elements Revealed
Toluidine Blue	Toluidine Blue	Nucleus (blue) and cytoplasm (light blue).Shows general structure, completion time is a few minutes
H&E	Haematoxylin and eosin	Most common histology method for general purpose. Nucleus (blue), cytoplasm, connective tissue (pink-red)
PTAH	Phosphotungstic acid hematoxyli	Reactive Astrocytes (blue)
Martius Scarlet Blue	Biebrich scarlet	Fibrin (yellow), collagen (blue), blood cells (red)
Masson's trichrome stain	Hematoxylin, Biebrich scarlet, acid fuchsnm, analine blue	Collagen (blue), nuclei (deep blue), blood cels and muscles, conective tissue (red), parenchymal tissue brain (glia) (pink)
Myelin	Mordanting followed by hematoxylin or Luxol blue	Myelin sheaths (blue)
Neuroglia	Cajal's gold sublimate or Hortega's silver sublimate	Astrocytes, oligondreocytes and microglia (black on brown background for Cajal's), Nerve cells (Red-Cajal's). Microglia (blue-Silver sublimate)

Neurons

Staining Method	Reagent	Elements Revealed
Nissl	Methylene blue or cresyl violet or hematoxylin. **Only Nissl and Myelin can be used in combination**	Nuclei of neurons, glia and blood vessels (blue)
Silver	Silver nitrate methods (Cajal, Bodian, Bielschowsky, Glees)	Nerve cell bodies and larger dendrites, axons and synapses. Body (yellow), axons and synapses (black)

Table 5-1. Histological Methods for CNS

Molecule	Description	Group	Reference
Neurofilament	Neurofilaments play an important role in the regeneration after SCI	Cellular element	[56]
GFAP	Protein specific for astrocytes and ependymal cells in the CNS	Cellular element	[56]
Serotonin	Modulates the central pattern generator for locomotion	Neurotrans mitters	[197]
GABA	Modulates synpatic transmission for interneurons	Neurotrans mitters	[197]
GAG	Glycosaminogylcan (GAG) is a part of most CSPGs used for localization	Scar (CSPG)	[198]
Netrin-1	Myelin-associated protein found to affect axonal growth in CNS	Scar (myelin-associated protein)	[44, 45]
NG2	Chondroitin sulfate proteoglycan (CSPG). Increase after stab injury and column transections	Scar (CSPG)	[195, 196]
Nuerocan	CSPG. Increase after stab injury and column transections	Scar (CSPG)	[198]
Phosphacan	CSPG. Increase after stab injury and column transections	Scar (CSPG)	[198]

Table 5-2. Immunostaining antibodies

Molecule	Description	Group	Antibody	Reference
Neurofilament	Neurofilaments play an important role in the regeneration after SCI	Cellular element	Mouse IgG1 mAb	[56]
GFAP	Protein specific for astrocytes and ependymal cells in the CNS	Cellular element	GFAP antibody (Rabbit)	[56]
Olig1	Specific for oligondreocytes in the nervous system	Cellular element	Goat IgG pAb	[56]
von Wilebrand factor	Labels endothelial cells	Cellular element	Rabbit pAb	[56]
Hoescht	Stains for living cell nuclei	Cellular element	Blue-fluorescence	Sigma-Aldrich
Serotonin	Modulates the central pattern generator for locomotion	Neurotransmitters	Anti-body for neurotransmitter	[197]
GABA	Modulates synpatic transmission for interneurons	Neurotransmitters	Anti-body for neurotransmitter	[197]
Dopamine	Modulation of spinal neurons and synaptic potentials	Neurotransmitters	Anti-body for neurotransmitter	[197]
Calcitonin	Modulation of serotonergic system, calcium homeostasis, pain relief	Neuropeptide	Rabbit antibody	[199]
Galanin	Inhibitory neuropeptide, increased during axotomy, involved in neural diseases.	Neuropeptide	Rabbit antibody	[199]
NG2	Chondroitin sulfate proteoglycan (CSPG). Increase after stab injury and column transections	Scar (CSPG)	Rabbit anti-G2	[195, 196]
Nuerocan	CSPG. Increase after stab injury and column transections	Scar (CSPG)	Mouse anti-neurocan	[198]
Phosphacan	CSPG. Increase after stab injury and column transections	Scar (CSPG)	Mouse anti-phosphacan	[198]
GAG	Glycosaminogylcan (GAG) is a part of most CSPGs used for localization	Scar (CSPG)	Mouse anti-CSPGs (CS-56)	[198]

Netrin	Netrin 1 is a myelin associated protein found to affect axonal growth in CNS of humans, rats and lampreys	Scar (myelin-associated protein)	Netrin-1 Chick antibody	[44, 45]
Nogo	Inhibitory molecule of the CNS	Scar (myelin-associated protein)	Nogo-A rabbit pAb	[21]
MAG	Inhibitory molecule of the CNS	Scar (myelin-associated protein)	MAG (chick) mouse IgG1 mAb	[22]
Fibronectin	Adhesion protein	Extracellular matrix (ECM)	Anti-body for fibronectin	[56]
Collagen	Adhesion protein	ECM	Anti-body for collagen	[56]
Laminin	Adhesion protein	ECM	Anti-body for laminin	[56]
Keratin	Adhesion protein	ECM	Anti-body for keratin	[56]
Chondroitinase	Bacterial enzyme that promotes functional recovery of spinal injury	Bacteria	Chondroitin ABC	[200]

Table 5-3. Complete antibody table for CSPGs, myelin-associated protein, adhesion molecules, neurotransmitters and neuropeptides, and cellular elements in the spinal cord.

5.2.3 Coordination tests for other SCI treatments

Treatments for spinal cord injuries (SCI) have greatly evolved with the use of molecular and cellular therapies. The use of stem cell therapy [201-206], targeted antibodies [207-210] and enzymes [211, 212] designed to control the immune response are some examples of molecular methods which have emerged as possible methods of treatment for SCI. Although not readily available for human use, these treatments have improved the neurological outcome of rodents [207, 211-213]. However, most results were effective for only a brief time window immediately after SCI [214-217]. In some cases, it may be necessary to use surgical techniques to enhance the effect of molecular therapies and prevent malfunctions of spinal circuitry.

In combination with molecular therapies, other techniques include the use of scaffolds [109, 213, 218-221], non-stem cell transplants [222, 223] and different surgical strategies [224], such as spinal decompression [225-229]. Similarly to molecular treatments, patients with surgical decompression done early after injury had an improved neurological outcome [224, 226]. Therefore, some researchers have concentrated in the use of imaging techniques, such as MRI, to better diagnose the damage after SCI in a prompt and effective manner [226, 230-233]. Despite the numerous advancements made in these fields, there still remains no way for humans to regain nervous function after complete SCI [234].

We have learned that the lamprey is an animal model that can be used to test the functionality of regeneration. We used clot removal to measure whether this

technique will improve the behavior of maladaptive animals. This same approach could be used for other treatments, such as the use of chondroitinase enzyme to degradate inhibitory molecules or the use of scaffolds to promote axonal regeneration. Researchers could use intraspinal injection of antibodies or enzymes for an inhibitory molecule on animals injured at warm and cold temperature (23 °C and 10 °C). Then, animals could be euthanized and the cord will be sectioned for immunostaining studies or we could use a time frequency analysis to leave the animal alive and observe their regeneration as a function of time.

Scaffold implantation could be used to investigate the development of a guidance tube or a porous hydrogel to be injected in situ after injury. Collagen gels could be investigated; other options are available such as alginate, agarose, hyaluronic acid, PEG and others [25]. In order to elaborate the scaffolds, investigators could use the mechanical properties of uninjured or adaptive animals that were studied in this thesis, so that the environment is permissive for regeneration.

Collagen has been use to promote axonal growth in rats [235] and to reduce scarring in human spinal laceration [236]. Collagen offers biocompatibility and natural cell adhesion; however, due to the extreme changes in pH and temperature needed for their cross linking, they are unsuitable for use as injectable gels. Therefore, gels could be done in a mold similar to the neural tube of the lamprey. New preparation techniques, such as the use of enzymatic crosslinkers or fibrillogenesis could make it possible to use gels in situ [237]. After scaffold implantation, a time frequency analysis

could be done to test animal coordination with different gels and observe which gel promotes the most effective recovery.

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