A Zebrafish Model of Spinal Cord Injury
Locomotor Deficits, Axonal Regeneration and Recovery of Function

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Abstract

Spinal cord injuries (SCI) can result in nearly complete loss of function below the spinal-level of damage, due to the interruption of descending neural commands. While a number of approaches are being tested to enhance axonal regeneration and assess the recovery of spinal cord function (primarily in rats), to date no effective regeneration has been demonstrated following complete SCI. In recent years, the larval zebrafish has become a popular organism for studying both basic spinal mechanisms and descending motor pathways. The larval zebrafish possesses nearly 300 neurons that extend axons from the brain into spinal cord. These descending neurons, including reticulospinal, vestibulospinal and other cell types, can be individually identified and visualized in intact animals. Because fish neurons show enhanced regenerative capabilities relative to mammals, this offers an attractive preparation with which to examine regenerative processes. We propose to use novel lesioning techniques to disrupt these descending pathways, after which regenerative processes will be followed both anatomically and behaviorally. Importantly, the larval zebrafish exhibits a broad range of locomotor behaviors (Budick and O’Malley, 2000; Borla et al., 2002; McElligott and O’Malley, 2005) which can be evaluated in both lesioned and recovering larvae. We will first establish a locomotor battery that assesses a broad range of motor functions ranging from the simplest swimming movements to the highest performance escape behaviors. Using this battery, we will next establish the degree of locomotor deficit resulting from different kinds of lesions, including labeled-lesions which disconnect large numbers of descending neurons and more precise lesions resulting from the in vivo cutting of axons using a pulsed laser. These experiments involve collecting confocal and two-photon image stacks to identify those neurons that have been disconnected from their spinal targets. We will then observe the cut axons in vivo to determine the extent and degree of axonal regeneration that occurs after the two types of lesions. Finally, we will characterize the degree of functional recovery that occurs in parallel with any observed axonal regeneration. Additional experiments are described that explore the potential utility of nanotechnology for the repair of spinal cord damage.
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A. Specific Aims

AIM 1: Create a locomotor battery for the larval zebrafish. Locomotor batteries are commonly used to assess performance of spinal-injured animals. We will establish a locomotor battery that efficiently characterizes the ability of larval zebrafish to achieve a range of motor performance benchmarks, as assessed by high-speed imaging of larval locomotor behaviors.

Goal: The locomotor battery will assess the degree to which larvae can perform a range of turning and swimming behaviors. The battery must be simple and reproducible, but also sensitive to behavioral deficits ensuing from the lesioning methods described in Aim #2. Larval motor patterns that are readily elicited include escape behaviors, light-evoked swims, dark-evoked turns, rheotaxis and the optomotor response (OMR). Based on our lab’s previous experience in analyzing these behaviors, we expect that a comprehensive battery of these functions can be performed in ~2 hours. This battery will establish kinematic performance measures that can be used in characterizing both locomotor deficits and the recovery of function after labeled-lesion and 2-photon axon cutting experiments. Moreover, the baseline measurements obtained with control fish (for our wild-type and Nacre zebrafish strains) will provide a foundation for future investigations that assess mutation-related deficits, optogenetic and molecular manipulations and the behavior of zebrafish used as neurological disease models.

AIM 2: Perform two-photon laser axotomies and labeled-lesions on zebrafish larvae and assess their functional consequences using the locomotor battery. Relate lesion size and location to the degree and nature of locomotor impairments. Perform appropriate controls (muscle-only and spinal-interneuron only lesions). We will also attempt to identify spared neurons to assess the locomotor capabilities of spared motor pathways.

Hypothesis 1: Varying degrees of locomotor deficits may result from substantial or minute alterations to elements of the descending motor control system (DMCS). The extent and location of neural impairment may also indicate how neurons of the DMCS contribute to the accurate timing and completion of differing locomotor gaits and patterns.

Hypothesis 2: Both small and substantial perturbations of the descending motor control system will result in minimal disruption of locomotor performance, suggesting possible organizational redundancy within the larval zebrafish DMCS.

In this Aim we will perform small precise lesions using two-photon cutting of either single axons or small axon bundles descending from brainstem. We will also create a broader range of lesion sizes and types using the labeled-lesion approach where a micropipette severs axons and simultaneously labels the damaged cells with fluorescent tracer. This array of lesion types is expected to reveal organizational features of the DMCS. Unilateral axotomies may e.g. produce selective effects on turning behaviors including routine and escape turns. Important controls will assess whether muscle damage and/or injury to rostral or caudal spinal interneurons are responsible for any of the observed locomotor deficits.

AIM 3: Visualize the time-course and extent of regeneration of labeled descending axons after labeled-lesion and laser-axotomy experiments.

Hypothesis 1: Descending regenerating axons will traverse directly through the lesion site to reconnect with spinal cord.

Hypothesis 2: During the process of regeneration, axon extensions will circumvent the lesion site to follow alternate routes to reach appropriate target sites.

Hypothesis 3: Sprouting of branches from spared axons, below the lesion site, will enable new connections to uninjured spinal neurons.
Though previous studies (Bhatt et al., 2004; Becker et al., 1997) have provided evidence in zebrafish of spontaneous neuroregenerative processes, there is a limited understanding of the extent and functional impact of spontaneous regeneration. By combining the results of the labeled-lesion technique, which generally creates large-scale lesions, with the more precise two-photon laser axotomy results, we hope to acquire a better understanding of the scope and timing of events that occur during larval axonal regeneration. In particular, this study will focus on observing how particular subsets of descending fibers respond to injury and how they may be able to reinnervate appropriate spinal target regions. In addition, the two-photon laser axotomies allow us to sever descending axons at precise locations and with minimal damage to the surrounding tissue. This technique may thus improve visibility at the lesion site and our ability to characterize the regenerative process.

**AIM 4:** Quantify the recovery of locomotor performance after labeled-lesions and laser axotomies. These experiments will rely on the locomotor battery established in **Aim #1**. We will also correlate the degree of functional recovery with the extent of axonal regeneration observed in **Aim #3**.

**Hypothesis 1:** Zebrafish larvae will recover some but not all of their locomotor capabilities.

**Hypothesis 2:** The degree of locomotor recovery will directly correlate with the extent of axonal regeneration.

**Hypothesis 3:** There will be varying degrees of locomotor recovery and they may not be well-correlated with axonal regrowth.

We will assess functional recovery based upon the benchmarks established in **Aim #1**. While there are reports of zebrafish, goldfish and lamprey recovering locomotor function, those studies are limited in scope and anatomical detail. The larva’s extensive locomotor repertoire, in conjunction with our ability to precisely define the lesioned populations, should further our understanding of the capacity and potential limitations of the regenerative process in larval zebrafish. A better understanding of how regenerating descending pathways contribute to functional recovery has broad implications for spinal cord injury research.

**AIM 5:** Conduct Pilot Experiments pertaining to the Potential Applications of Nanotechnology to Spinal Cord Injury Research.

**Hypothesis 1:** Gold nanoparticles transported by retrograde mechanisms in the larval zebrafish will indicate the potential for targeted transport of therapeutic compounds to the cell bodies in the hindbrain.

**Hypothesis 2:** Primary cultured hippocampal neurons will reliably adhere to a nanowire interface and remain viable for a defined time frame.

Efforts in the field of spinal cord injury to develop macro/nanoscale tools to repair axons have until now followed two approaches. One is the development of new nanoscale tissue scaffold materials to act as bridges to support and stimulate axon regeneration. The other is the use of novel cellular-scale surgical micro/nanodevices designed to perform surgical inter-weaving towards the repair of severed axons. Our pilot experiments have explored the potential for (1) the retrograde delivery of nanoparticles to disconnected neurons and (2) the feasibility of neural-nanowire array interfaces for their potential utility for induction of axonal regeneration and/or as a means to implement nanoprosthetic devices. While this is an interesting avenue of research, our planned future work is encompassed by **Aims #1 through #4**, focusing on the biological questions of interest.
B. Introduction

B.1 General Problem of Spinal Cord Injury

Spinal cord and brain injuries resulting from trauma or disease pose challenging medical issues that are often insurmountable, leading to chronic disabilities and death. Major goals of the neurological research community aims are thus to minimize the neural damage that occurs in these types of injuries and to repair the damage by induction of neurogenesis and self-repair mechanisms and by other means. However, there are formidable barriers to repairing damage in the mammalian central nervous system (CNS) due to such reasons as: 1) scar tissue formation after injury; 2) gaps in the tissue due to phagocytosis; 3) inhibitory factors that impede axonal growth in the mature CNS; and 4) the failure of most neurons to initiate axonal extension (Kwon et al., 2001; Silver and Miller, 2004; Thuret et al., 2006). Unfortunately this is not by any means a complete list.

Spinal Cord Injuries (SCIs) are unique in a number of respects, including their immediate impact on the victims, sometimes leaving them completely paralyzed. They are also unique in that the damage is typically localized to a narrow segment of spinal cord, involving in severe cases a complete crush or severing of the connections between the brain and the spinal cord below the lesion site. Extensive research indicates that substantial reorganization of the lesion site and surrounding nervous tissue occurs, as found both by analysis of pathology and in experimental studies. Whether this occurs as a result of 1) alterations to pre-existing circuitry along the lines of synaptic plasticity or 2) the formation of essentially new circuits as a result of more extensive anatomical reorganization (Raineteau and Schwab, 2001), there appears to be substantial opportunity for modification of the motor control system in mammals. For example, a study in cats in which descending fibers to spinal cord were cut by spinal transection showed that these cats were capable of limited locomotion on a treadmill at one month post injury (de Leon et al. 1998). In these animals afferent feedback, or information directed from peripheral nerves to the spinal cord, is needed for the recovery and correction of stepping movements. This type of functional recovery was observed in peripheral nerve lesioning experiments performed on cats following denervation of the hind leg muscles (Pearson and Misiaszek, 2000).

Because of the focal nature of SCI, it offers an attractive target for interventions seeking to repair neural damage or work around a lesion site. In cases of complete spinal transection, recovery of normal locomotor function requires that descending axons from the brainstem traverse the lesion and restore control over the lower spinal cord networks. Much of this work has focused on studies in rodent models which are intended to provide results that can be translated into human treatments. Many studies have used growth factors to try and stimulate regeneration of axons from brain into spinal cord (Giger et al., 2010; Kasai et al., 2010; Yang et al., 2010). Other studies have sought to counteract inhibitory factors such as Nogo (Harvey et al., 2009; Wang et al., 2010; Wu et al., 2010). A third strategy is to use glial cells, such as olfactory ensheathing cells, to promote axonal regeneration (Kocsis, 2009; Lindsay et al., 2010; Raisman et al., 2010). Alternatively, engineering approaches can be employed to route neural signals around the site of injury e.g. using bridging grafts (Tabesh et al., 2008; Wong et al., 2008; Zurita et al., 2010). Perhaps one day neural implants will be used as is now the case with cochlear implants; more novel devices are currently being tested in cortex and retina. We have done exploratory studies in this latter area to assess the possibility of using nanowire arrays as a brain-machine interface through which neural commands could be either picked up above the lesion site or delivered below the lesion site to restore spinal cord function, as discussed below. Another approach in the nanotechnology realm is the use of nanoparticles to deliver therapeutic compounds including e.g. growth-promoting genes. Pilot experiments of ours along these lines are also discussed below. But despite the efforts of many research groups, there has been, to date, no effective progress in restoring locomotor function in humans following complete spinal-cord transection. The best results to date have involved rehabilitative treatments and
neuroprotective/growth factors, but these with only limited success (Thuret et al., 2006). This is a formidable problem, not simply because of the biological barriers to axonal regeneration, but also because we are lacking basic knowledge about the numbers and types of descending connections required either for normal locomotor functions or to restore function after SCI. Contributions from simpler model systems are potentially beneficial in both regards.

Role of Neurogenesis and Axonal Regeneration. In the mammalian CNS, functional neurogenesis is restricted primarily to the olfactory system via the rostral migratory stream, along with an apparently more limited neurogenic process in the hippocampus (Eriksson et al., 1998; van Praag et al., 2002; Ghashghaei et al., 2007). In contrast, many lower vertebrates display a much greater capacity to continually produce neurons through adulthood. We should note that adult neurogenesis and axonal regeneration are present in many amniotes, which include fishes and amphibians, and that this clade of organisms has provided a variety of insights into the regenerative processes of the CNS (Windle, 1956; Bernstein and Gelderd, 1970; Rovainen, 1976, Davis et al., 1990; Becker et al., 1997; Chernoff et al., 2003; Chevallier et al., 2004; Zupanc and Zupanc, 2006; Kaslin et al., 2008; Kragl et al., 2009). Studies in lower vertebrates are thus of potential benefit, both in terms of understanding the genesis and reorganization of neural circuits following SCI and in terms of understanding molecular differences that enable them to naturally respond to injury. Of particular interest are vertebrate models shown to have axonal regrowth and functional recovery, as they may be useful in determining the mechanisms necessary for functionally-relevant regeneration. In the next section we summarize some of the results obtained with certain models and the extent of functional recovery attained. Our focus is on the collective pathways from brain to spinal cord—the descending motor control system (DMCS). We are particularly interested in the regenerative potential of individually-identified descending neurons that make up the reticulospinal, vestibulospinal and other pathways that project from brain into spinal cord.

B.2 Lower Vertebrate Models

It is commonly assumed that lower vertebrate animals “regenerate” damaged spinal cords and recover locomotor functions. An accompanying notion is the idea that the regenerated descending axons reconnect to their normal spinal targets. But a careful read of the results obtained after spinal cord transection in lower vertebrates reveals that these ideas are often overstatements and may be incorrect in several important respects. While amphibians and fishes clearly show greater regeneration than mammals, all vertebrate animals have some limited capability to respond to neural damage by such means as neurogenesis, axonal sprouting, axonal regeneration and functional reorganization (Raineteau and Schwab, 2001; Thuret et al., 2006). But all vertebrate animals are also limited in their ability to regenerate descending spinal axons past a glial scar (Silver and Miller, 2004) into undamaged lower spinal cord. To clarify this situation, we summarize below a number of key findings on the regenerative potential of several well-studied model organisms.

Studies on Larval Lamprey

Recovery of descending motor control, in the case of spinal transection, requires that axons descending from the brainstem traverse the spinal lesion and restore control over caudal spinal locomotor networks. Among lower vertebrates, the lamprey has the best documented regenerative capabilities (Table 1) wherein a variety of locomotor functions are recovered following complete spinal cord transection (Windle, 1956; Ayers, 1988; Jin et al., 2009). Larval lamprey in particular were reported to recover such locomotor functions as swimming, coiling and crawling (Rovainen, 1976). They also exhibit several stages of recovery in which they obtain progressively better-coordinated swimming and other behaviors (Ayers, 1988). For example, functional regeneration of the L1 “fin-command” neuron was reported in that strong stimulation of L1 could control fin posture after 80 days of regeneration (Currie and Ayers, 1987). McClellan reported the first regeneration of “command” neurons in lamprey (1988, 1990) in that...
stimulation of brainstem could evoke fictive locomotor activity in the in vitro brain/spinal cord preparation and this appeared to be mediated by a lateral swim-command pathway. Accompanying these behavioral recoveries, numerous anatomical studies have shown varying degrees of regeneration of descending axons (Rovainen, 1976; Selzer, 1978; Wood and Cohen, 1979; Davis and McClellan, 1994). One double-label study reported that 70% of brainstem neurons regenerate their axons at least 10% of the body length beyond a rostral-spinal lesion site (Zhang and McClellan, 1999). Roughly 90% of those axons that do regenerate after spinal transection are observed projecting in the correct direction, including both large descending axons and spinal interneurons (Mackler, Yin, Selzer, 1986).

There are, however, substantial limits to lamprey regeneration. Most notable is the frequent failure of neurons, including the Mauthner and Muller neurons, to properly regenerate: their regenerating “neurites” may instead branch and project aberrantly by e.g. looping backwards (Yin and Selzer, 1983; and see Becker et al., 1997). Yin and Selzer noted that after spinal transections “all cell types examined thus far...have a limited capacity for axonal regeneration.” In this study, the regenerating axons failed to regenerate significant distances into spinal cord, projecting on average much less than 1 mm beyond the site of the spinal transection, as estimated from intracellular HRP fills. Moreover, the number of branches was reported to drop late in the regeneration process (Yin and Selzer, 1983). In other studies, however, longer-range regeneration was reported, reaching 40% of body length for 27% of regenerating axons, and in 5% of axons regenerating up to 60% of body length (Davis and McClellan, 1994). But regenerated axons failed to re-establish normal patterns of synaptic connections in spinal cord, as indicated in ultrastructural studies where the number of presynaptic boutons was greatly reduced (Oliphint et al., 2010). Based on these and other studies we can conclude that the “regeneration” does not entail a normal degree of reconnection of descending axons with their normal spinal targets, especially since the normal targets typically are much further down spinal cord than the regeneration distances observed.

Regeneration of correct intraspinal synapses below the lesion site has been reported (Mackler and Selzer, 1987) and this might lead to functional recovery via the “propriospinal” system—the long-range intraspinal projection neurons. Such pathways are suggested to account for locomotor recovery beyond the anatomical reach of regenerating axons (Davis et al., 1993). Thus some combination of axonal regeneration and rearrangement of connectivity, possibly utilizing propriospinal neurons, may contribute to a reorganizational mechanism of locomotor recovery, as is also believed to occur to some degree in mammals, albeit to a much more limited extent (Raineteau and Schwab, 2001; Thuret et al., 2006).

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<th>Focus of Research</th>
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| Locomotor Recovery/Functional Regeneration | Shibata et al. (2000)  
|                                        | McClellan et al. (2009)  
|                                        | Mecacci and McClellan (1993)  
|                                        | Cavia et al. (1997)  
|                                        | Li and Selzer (1991)  
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|                                        | Solzich and Selzer (1999)  
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|                                        | Cohen (1989)  
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|                                        | Selzer et al. (1980)  
|                                        | Rovainen et al. (1997)  
|                                        | Wende et al. (1989)  
|                                        | Cook and Parker (2009)  
|                                        | Zhang et al. (2006)  
|                                        | Amat et al. (2005)  
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|                                        | Cavia and McClellan (1993)  
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|                                        | Yin and Selzer (1985)  
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|                                        | Yan et al. (1987)  
|                                        | J.M. et al. (2009)  
|                                        | Oliphant et al. (2010)  
|                                        | Cohen and Hall (1990)  
|                                        | Zhang et al. (2005)  
|                                        | Pyke et al. (1989)  
|                                        | Lane et al. (1994)  
|                                        | Lane and Selzer (1991)  
|                                        | Mectler et al. (1989)  
|                                        | Yan et al. (1985)  |

| **Regeneration of axonal tracts beyond the anatomy** | | |
| **Responses in spinal interneurons following anatomy** |
| Promoting axonal regeneration | Synaptic connections |
| Degenerate and axonal sprouting following anatomy |
| Growth of axons and neurite tips |

**Table 1.** Studies performed in larval and adult lamprey relating to axonal regeneration of brainstem neurons and the level of functional recovery.
**Adult Lamprey**

The repertoire of adult lamprey behaviors is more extensive than exhibited by larvae and has been assessed in a spinal regeneration context (Ayers, 1988). Kinematic analyses that focused on parameters central to the flexion waves that characterize undulatory swimming were able to distinguish a broader range of behaviors than those exhibited by the filter-feeding larvae. These adult behaviors included escape and normal (navigational) swimming and other locomotor behaviors such as “terrestrial” swimming, crawling, burrowing, struggling and aversive withdrawal. Adult lamprey appear to have similar regenerative capabilities as larval lamprey in that substantial numbers of descending neurons regenerate axons past the lesion site into the distal spinal cord, with most neurites oriented in the correct direction and running along ipsilateral spinal cord (Lurie and Selzer, 1991).

In summary, both larval and adult lampreys have substantial limits to regeneration, but also substantial regenerative capabilities. There are some striking similarities between the rat and lamprey models, as e.g. a recently documented distinction between growth-cone mediated (developmental) and growth-cone independent (regenerative) growth mechanisms in lamprey (Jin et al., 2009; and see Bhatt et al. 2004) and a “2 mm limit” for regrowth of axons into healthy tissue (Yin and Selzer, 1983), both of which may apply to lamprey and higher vertebrates alike. It also seems clear that cAMP facilitates regeneration and that this proceeds via a later developmental mechanism that may be independent of growth-cone actin dynamics, relying instead upon propulsive forces probably involving intermediate filaments (Jin et al., 2009). The lamprey model has thus been a rich source of information with implications for regeneration in mammals, teleost fishes and other higher vertebrates. Complementary to this is the quite recent emphasis being placed on understanding the precise neural circuits and behaviors of the larval zebrafish. After reviewing some of the historical adult zebrafish and goldfish regeneration literature, certain benefits of examining regeneration in larval zebrafish are proposed.

**Adult Zebrafish and Goldfish.**

While lampreys exhibit a variety of swimming and postural behaviors, teleost fishes have a richer locomotor and behavioral repertoire, although there has not been to our knowledge an effort to describe a complete “ethogram” of behaviors for teleost fishes or higher vertebrates. Historically, the next best studied regeneration model has been the goldfish, *Carassius auratus*. To the extent characterized, teleost fishes appear to have similar capacities and limits to their regeneration as lamprey (Table 2). “Normal” swimming appears 25 days after spinal transection of adult goldfish, and by 60 days, up to 50% of descending fibers have regenerated 2 cm into the distal spinal cord (Bernstein and Gelderd, 1970). Curiously, the regenerating fibers were quite swollen, between 4 and 8 times their normal size, which is in contrast to the abnormally thinner diameters of regenerating lamprey fibers (Oliphant et al., 2010). The regenerating neurons included reticulospinal, vestibulospinal and nMLF neurons (Coggeshall et al., 1982), and an HRP labeling study showed that some regenerating axons could extend almost the length of the spinal cord (Coggeshall and Youngblood, 1983). A recent study claimed that regenerating axons from the nucleus of the medial longitudinal fasciculus (nMLF) re-establish synapses to “correct” spinal targets after a partial spinal cord lesion (Takeda et al., 2007), but the specific spinal targets of these nMLF neurons has not been determined (Gahtan and O’Malley, 2003).

There are certainly varying reports on the extent of regeneration, with one study noting great variability in regeneration of different fiber tracts (Bunt and Fill-Moeb, 1984). The degree and specificity of axonal targeting by descending neurons following an insult to the system also remains unknown. Early work showed that after spinal transection, many of the connections from goldfish RS neurons onto spinal neurons were lost and were replaced by synapses from spinal interneurons (Bernstein and Gelderd, 1973). A more recent study of adult goldfish reported that only 11 of the 17 brainstem nuclei that normally project into spinal cord are able to do so after spinal transection (Sharma et al., 1993), and the degree of
functional recovery is also incomplete, and limited by incorrect choices of pathway by fibers that are regenerating (Bentley and Zottoli, 1993). More detailed behavioral analysis showed frequent failure to recover normal posture and absent or abnormal C-start escape behaviors (Zottoli and Freemer, 2003). These findings are in accord with studies in lamprey, where there is limited axonal regeneration and even more limited re-establishment of synaptic connections to the original spinal targets. In both cases, engagement of intraspinal networks appears to be important for the limited recovery of the locomotor repertoire.

Table 2. Partial list of studies performed in goldfish and zebrafish on axonal regeneration of brainstem neurons and expression of intrinsic factors

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<td><strong>Goldfish</strong></td>
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<td>Regeneration of long fiber tracts beyond the axotomy</td>
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<td>Selective regeneration of brainstem nuclei</td>
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<td>Trajectory of regenerating axons</td>
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<td>Degree of functional recovery post axotomy</td>
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<td><strong>Adult Fish</strong></td>
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<td>Selective regeneration of brainstem nuclei</td>
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<td>Trajectory of regenerating axons</td>
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<td>Molecular response to axotomy in adult fish</td>
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<td><strong>Larval Fish</strong></td>
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<td>Promoting axonal regeneration</td>
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<td>Locomotor deficits following axotomy</td>
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As the zebrafish has gained traction as a model organism, a number of studies have examined axonal regeneration in adult zebrafish (Becker and Becker, 2008). Becker et al. (1997) reported that most but not all of the 20 different descending nuclei are able to regenerate axons into distal spinal cord after spinal transection. These tracts re-route to gray matter below the lesion site, while the adjacent white matter showed extensive myelin debris (Becker and Becker, 2001). As befitting the suitability of zebrafish for molecular studies (Goldman et al., 2001), the Becker group has also explored a number of molecular aspects of spinal regeneration, showing for example that some descending neurons upregulated their expression of the growth promoting genes GAP-43 and L1.1 and regenerated after spinal transection, whereas ascending spinal-brainstem neurons showed no such molecular response and concomitantly failed to regenerate (Becker et al., 2005). Morpholino knockdown of L1.1 impaired both axonal regeneration and locomotor recovery (Becker et al., 2004). In addition, a zebrafish homolog of mammalian contactin1 was found to be upregulated in both axotomized descending neurons and spinal
white matter glial cells (Schweitzer et al., 2007). Expression of other cell recognition molecules such as NCAM and L1.2 were examined in several brain regions after distal spinal lesions, but there were no expression responses associated with spinal regeneration (Becker et al., 1998).

Because zebrafish and goldfish are closely related otophysan species, the cumulative anatomical results on regeneration are understandably similar, but they also are complementary to and reinforce conclusions from lamprey regarding the limits of spinal regeneration. We have, however, less information on the “locomotor recovery” of adult otophysan swimming, as this has been described mainly to the extent of the claim that swimming is “normal”, but without any definitive high-speed kinematic analyses that might distinguish different types of locomotor behavior. One clear conclusion from the adult studies is that the swimming fatigues very easily, suggesting that the muscles are used in a less-efficient manner which might reflect subtle abnormalities to the swimming pattern that are not detectable from visual or video-rate observations. Zebrafish larvae are known to have a surprisingly large and specialized locomotor repertoire, and it seems only reasonable that adult zebrafish will have an even larger and more sophisticated repertoire. While this has been documented for several forms of social-locomotor behaviors (Larson et al., 2006; Imada et al., 2010), the adult locomotor repertoire has not been examined in any kinematic detail, beyond the much examined escape behavior (Foreman and Eaton, 1994; Zottoli and Freemer, 2003).

From Fidelity and Extent to an Operational Approach to Vertebrate Spinal Regeneration

It would be quite useful to know how the fidelity and extent of axonal and synaptic regeneration correlate with behavioral recovery, but this currently cannot be known with certainty in any vertebrate animal. The model systems reviewed here attempt to quantify the number of successfully regenerating fibers and the degree to which they re-establish correct synapses, but run into serious limitations of our basic knowledge of the normal descending pathways. Many descending nuclei do indeed contribute to regeneration, but these nuclei are heterogeneous and generally comprised of indeterminate numbers of neurons, even though the neurons are in principal individually identifiable, as has been documented for both adult goldfish and adult zebrafish (Lee and Eaton, 1991; Lee et al. 1993), as well as the lamprey (Rovainen, 1976). Thus, we do not know which identified neurons regenerate, nor the extent to which they reconnect with either their normal spinal targets or with other individually-identified spinal motoneurons or interneurons (see e.g. Liu and Westerfield, 1988; Bernhardt et al., 1990). This inability to define the precision with which synaptic contacts are re-established after regeneration applies even more so to mammals, since we are far from being able to define the complement of descending and spinal neurons that normally participate in mammalian locomotion. Indeed, even in the case of the simplest of vertebrate model systems, such as the larval zebrafish, where the descending and spinal neurons are individually identified (Liu and Westerfield, 1988; Hale et al., 2001; O’Malley et al., 2003), we still do not know the precise spinal targets of virtually any descending neuron in control animals, which makes (at present) determining the fidelity and extent of regeneration impossible. This situation is expanded upon below, in the context of our proposed alternative approach, which is to gain a better understanding of the causal effects of disconnecting defined sets of descending neurons, and the recovery processes that occur both anatomically and behaviorally.

B.3 The Larval Zebrafish Model

The transparency of larval zebrafish and identifiability of the descending motor neurons makes this animal model a candidate for a more comprehensive investigation into neural regeneration and repair processes. In order for efforts along these lines to be successful, a good deal of foundational work is required. The locomotor repertoire of larval zebrafish has been intensively studied, demonstrating a great variety of distinct behaviors, which can serve as benchmarks for studies of regeneration. These include
the escape behavior (Kimmel et al., 1974), routine turning and swimming behaviors (Budick and O’Malley, 2000; Thorsen et al., 2004), predatory behaviors (Borla et al., 2002; McElligott and O’Malley, 2005) and navigational behaviors (Burgess and Granato, 2007a [JEB]; Orger et al., 2000; Sankrithi and O’Malley, 2010). There has been a corresponding emphasis on understanding the neural basis of many of these behaviors (O’Malley et al., 1996; Liu and Fetcho, 1999; Ritter et al., 2001; Gahtan et al., 2002, 2005; Burgess and Granato, 2007b; Orger et al., 2008), which include calcium imaging and laser-ablation experiments directed at individually-identified neurons. The anatomical basis for these behaviors was described many years ago (Kimmel et al., 1982; 1985) and has recently been summarized (O’Malley et al., 2003) and is shown in FIGURE 1).

**Figure 1.** The Larval Zebrafish has ~300 Descending Neurons. (A) A confocal montage of the larval zebrafish neurons in the brain which includes reticulospinal and vestibulospinal neurons, neurons of the nucleus MLF (nMLF) and other neurons projecting into spinal cord. Identified in original HRP studies from the Kimmel lab [Kimmel et al., 1982, 1985; Metcalfe et al., 1986] (B) The Descending Motor Control System (DMCS) template which includes ~150 DNs on each side of the brain, and can be subdivided into 40 distinct cell types. Each box in the template represents an individual neuron, and many types are comprised of a single neuron, such as the Mauthner cell, MiD2cm and MiD3cm, and so are identifiable as exact individuals.

While there have been few studies of spinal regeneration in larval zebrafish, the targeted regeneration of the axon of the Mauthner cell was examined in what was perhaps the first study to visualize the process of axonal regeneration in an intact, living vertebrate animal (Bhatt et al., 2004). From the lamprey and teleost literature it has long been known that large, specialized descending axons, including the Mauthner and Muller cells, often follow incorrect paths during regeneration and this is also the case for larval zebrafish. Bhatt and coworkers, showed that the Mauthner cell could be induced to regenerate and restore escape-like behaviors by directly applying cAMP to the Mauthner cell. This presaged a study showing strong stimulating effects of cAMP on axonal regeneration in lamprey (Jin et al., 2009). However, we
still have only limited understanding of the regenerative capabilities of the larval zebrafish in terms of both anatomy and behavioral recovery, despite the increasing palate of genetic and optogenetic tools, and its increasingly well characterized set of neural systems in the larva.

Surprisingly, we also do not know the normal pattern of innervation of descending neurons onto spinal interneurons—even though these two populations are better defined in larval zebrafish than in any other vertebrate animal. There are at least 40 distinct classes or types of descending neuron, many of them individually identified (Figure 1) and at least 15 distinct types of spinal interneuron, along with 2 classes of spinal motoneuron including the large primary motoneurons RoP, MiP and CaP which are individually identified (Liu and Westerfield, 1988; Bernhardt et al., 1990; Hale et al. 2001). While spinal axonal trajectories have been visualized for many of the identified descending neuron types (Gahtan and O’Malley, 2003), there has not yet been any determination of their spinal targets, aside from the exceptional case of the Mauthner cell (Fetch and Faber, 1988). While this may seem a straightforward experiment, this data has not been acquired despite the many groups studying zebrafish brainstem and spinal cord. This is not a “zebrafish” problem, because as noted above, the connectivity of the DMCS has not been mapped for any vertebrate animal. Indeed, the cells that make up the most fundamental unit of the spinal cord, the swimming/walking CPG, have not been conclusively identified in any vertebrate animal despite many years of effort (Buchanan 2001, Gosgnach et al., 2006; Fetcho and McLean, 2010). The identities of the neurons that descend into the spinal cord are even more poorly defined, and absent an understanding of what neurons descend from brainstem and what specific sets of spinal neurons they synapse upon, it is impossible to construct a lower-motor system connectome.

Nonetheless, the zebrafish model remains the best available model, both in terms of the definition and accessibility of its DMCS and in terms of its kinematically well-defined locomotor repertoire. It thus offers an alternative approach to evaluating the spinal regenerative process, namely the prospect of disconnecting precisely (individually) defined sets of descending neurons from their spinal targets and relating these anatomical deficits to functional (behavioral) deficits. What is further unique for the larval zebrafish preparation is the capability to follow neuroanatomical regeneration in live animals and to quantify the precise extent to which “live regeneration” correlates with locomotor recovery. By examining the regenerative responses of individually-identified neurons we also hope to learn more about the variability of the regenerative process and how that relates to deficits and recovery potential. This may reveal anatomical details of the regenerative process that have not been detectable in other vertebrate animals. Given the great between-animal variability that is observed in adult fishes and lamprey (and which may also contribute to the muddied waters of mammalian regeneration), the finer grained approach possible with larval zebrafish should provide more precise results, potentially with novel insights that may serve as a guide to clinical researchers seeking to restore descending motor control in mammals. In addition, the partial lesions examined here may be more relevant to the large numbers of patients with incomplete spinal cord injuries.

B.4 Relation to Experimental Aims

The functional consequences of disconnecting defined sets of descending neurons from their spinal targets have yet to be systematically addressed in any vertebrate animal. As a preliminary step, we have created a locomotor battery that assesses a broad range of locomotor capabilities. This battery should therefore be sensitive to both obvious and subtle locomotor deficits (Aim #1). But the main focus of our research plan involves understanding how lesion size, location and cell-set correlate with the initial deficits observed (Aim #2) and with the ability of zebrafish larvae to recover key capabilities of their descending motor control system (Aim #4). Complementary anatomical information will be obtained by imaging the regenerative process in living zebrafish (Aim #3). We hope to observe fine details of the regenerative process including retraction, regrowth and the path taken by the regrowing axon/neurite, as well as the presence or absence of growth-cone like structures (Jin et al., 2009). This will be the first
visualization of individually-identified neuron regeneration inside a gnathostome vertebrate aside from the zebrafish Mauthner cell. For **Aim #3**, axonal regeneration will be observed after both spinal-injection based lesions (i.e. labeled lesions) and two-photon laser microaxotomy. Labeled-lesions produce large behavioral deficits involving large numbers of identified neurons, while two-photon laser axotomy provides a precise and relatively non-invasive methodology that appears to minimize injury to the surrounding tissues, thus allowing improved visualization of axonal regeneration. **Aim #3** combines synergistically with **Aims #2 and #4** in that we will be able to assess the degree to which behavioral deficits and recovery correlate with the initial lesions and with the axonal regrowth observed inside the spinal cord of these living animals. Because of the extensive locomotor repertoire, these comparisons will provide a sensitive, graded means of quantifying successively higher levels of locomotor performance for a diverse set of brain-guided behaviors—to a degree which has thus far not been accomplished for any vertebrate animal.

*Applications of Nanotechnologies- **Aim #5.*** We have also performed research supported by the NU IGERT Nanotechnology program which involved preliminary experiments exploring the potential of two nanotechnology methods for treating spinal cord injuries. The first involves visualizing the retrograde transport of functionalized nanoparticles, with a long-term goal of delivering neural repair genes specifically to sets of descending neurons that have been disconnected from their spinal targets. The second approach explored the feasibility of creating nanowire arrays that might provide a fine-grained interface with living neural networks; such arrays might in the future be implanted both above and below spinal lesion sites to serve as transmitters and receivers of locomotor commands. If successful, such approaches might be employed to treat a much broader range of neurological and neurodegenerative disorders.

**Aims #1 through #5** thus promise advances in terms of both technology development and advancing our understanding of basic aspects of brainstem-spinal circuitry and its response to varying degrees of injury. Success with any of these Aims would produce fundamentally new tools and knowledge; collectively they should provide a material advance of the zebrafish model of spinal cord injury. More importantly, this thesis will reveal many new biological facts about locomotor deficits, locomotor recovery and the response of individually-identified neurons to spinal axotomy.
C. Methods & Preliminary Data

C.1 Behavioral Analysis

The experiments described in this section constitute the locomotor battery being developed in Aim 1, with the ultimate goal of assessing the degree of locomotor deficit and recovery following an injury to the spinal cord and Descending Motor Control System (DMCS). The larval zebrafish exhibits a variety of complex and demanding locomotor behaviors, which can therefore be sensitive indicators of locomotor performance. Depending on the extent of the lesion resulting from either an injury via the micropipette axotomy or the 2-photon laser axotomy, locomotor behaviors being assessed will indicate the level of impairment and potential recovery. Two different modes of movement initiation will be tested: 1) mechanically and 2) visually-evoked. Kinematic parameters previously defined will also allow us to focus on particular analytic measures that will aid us in characterizing each animal’s locomotor performance [Budick and O’Malley, 2000; Borla et al., 2002, Burgess and Granato, 2007]. The experiments performed here establish baseline parameters as identified from control animals.

C.1.1 Behavioral Methods

Fish Husbandry
Adult zebrafish (wild type and nacre) were maintained for breeding purposes and housed in groups of 2 or less in 10 gallon tanks. The zebrafish were fed live nauplii of brine shrimp two times per day and TetraMin flakes (Melle, Germany). The water conditions of tanks were kept at pH 6.6 - 6.8 range and temperature of 28 - 29°C. To prevent accumulation of feces and maintain proper levels of nitrates and nitrites 20% water changes were performed weekly. Zebrafish strains used in this study included AB or Ekkwill (Ekkwill Fish Farms, Gibsontown, FL) and nacre [missing melanophores (black pigment cells)] (Moore, 1995 and Lister 1999). Embryos were produced by pairwise matings and raised at 28.5°C in 12% Instant Ocean (IO) salt solution. Fertilized eggs were collected from colonies of adult zebrafish, and maintained in an incubator at 28.1 - 28.5°C in IO water (proper pH and 2.4g Instant Ocean). Larvae were used (4 - 9 dpf) and raised on a 14-10 hour light-dark cycle. The 60 - 90% of the water was replaced daily and were fed paramecium from 5 days post fertilization (dpf) and nauplii of brine shrimp from 10 days post fertilization. All animal studies were approved by Northeastern University Animal Care and Use Committee.

High Speed Imaging
High-speed videos will be acquired using an EG & G Reticon circuit interfaced with a charge coupled device camera mounted on a Zeiss dissecting microscope, at acquisition rates of 600 frames per second. A fluorescent ring light was used to illuminate the field depending on the behavioral battery being tested. Videos were saved as AVI files and analyzed with ImageJ freeware (NIH).

C.1.2 Elements and Testing of the Locomotor Battery
Behavioral analysis will be performed on larva 4 - 10 dpf. Larva of this age have a relatively fixed locomotor repertoire [O’Malley et al. 2004], as well as a positive response to the OMR stimulus at this age [Orger et al. 2000]. The locomotor battery will consist of mechanical and visually elicited responses. In addition, we will collect behaviors that occur during these recording sessions for comparison with spontaneous behaviors exhibited by lesioned animals. The individual components of the battery are as follows: (1) touch elicited escape, (2) light/dark adaptation, (3) optomotor response (OMR), (4) rheotaxis, (5) slow and burst swims. All behavioral testing will be performed between 12 and 48 hours post injury for behavioral deficits and thereafter for any subsequent recovery up to 5 days post injury. Each fish will be tested twice at each time point for each task defined in the behavioral battery for consistency. Details of the battery components are described below:
Touch-Elicited Escape: Startle responses in teleost fish are mediated by reticulospinal neurons [Nissanov et al., 1990; Faber and Korn, 1978; Fetcho, 1992] which activates excitatory spinal interneurons and motoneurons. In larval zebrafish, the escape responses are initiated by a very fast C-shaped bend that is followed by a rapid, large counterbend in the opposite direction away from the stimulus [Kimmel et al., 1980; Foreman and Eaton, 1993; Liu and Fetcho, 1999, Budick and O’Malley, 2000]. This is followed by a vigorous swimming episode, referred to as a burst swim, following the counterbend. For our battery, larval zebrafish (4-10 days post fertilization) will be transferred into transparent 35mm circular dishes and exposed to a bright white LED ring light (intensity, approximately 8 W/m2, 1000 lux) for 5-10 min. The larval escape response (Fig. 2) is elicited with a light tap on the right or left side of the head with a small metal probe, after the larvae was allowed to acclimate in the dish for 10 minutes. An interval of at least 2 min and at most 10 min passed between trials.

Figure 2: Escape response. A gentle tap to the larva’s head elicits a C-start escape behavior consisting of a high angular velocity C-bend and a vigorous burst swim with substantial yaw. Video was collected at 400 frames/sec. 7 dpf EKW strain.
Light / Dark Elicited Behaviors: Zebrafish larvae have acute motor response as a result of an abrupt change in illumination. When lights are turned off, larvae have been observed to turn directly towards an extinguished light. Kinematic analysis of the visually-evoked turn have indicated that these are short duration, large angular velocity turns [Burgess and Granato, 2007; Day, 2006; Day, 2008]. Alternately, after lights are turned on, larvae in some instances turn before performing a slow swim. Analysis of the motor pattern of the slow swim and the light-evoked swim behaviors indicates that the mean bend angle, bend location and yaw remain unchanged [Day, 2008]. The experimental setup used to observe this behavior is described as follows. Larval zebrafish (4 -10 days post fertilization) will be transferred into transparent 35mm circular dishes and experiments carried out at 26 -28°C. An infrared LED (880nm peak) will be placed below the larvae, in view of the high-speed camera. The entire apparatus is then isolated in a heavy black shroud to prevent any stray light from penetrating the setup. As previous work indicates, the sensitivity to light drops after 620nm [Brockerhoff et. al., 1995] and therefore should not have any significant visual stimulation [McElligott, 2005]. The larva is allowed to acclimate for 10 minutes to the swimming arena lit with only the IR light (Edmond Optics) from below for light-evoked swim (LESs). Latency period will be measured for this behavior in order to exclude any responses that may have taken longer than 2 seconds, suggesting that the behavior may not have resulted from the light stimulus. For the dark-evoked turn, the larvae are exposed to a bright light source for 10 minutes (light adaptation). When a quiescent period is observed by the experimenter, the natural light will be turned on to initiate a light-evoked response (LES) (Fig. 6) and turned off to initiate a dark-evoked turn (DET) (Fig. 5). High-speed video recordings are initialized 300ms before the stimulus is presented and collected at 400 frames/sec.

Optomotor Response (OMR): The OMR can be elicited by either a rotating drum surrounding the larva [Cronly-Dillon and Muntz 1965; Clark 1981; Bilotta 2000; Krauss and Neumeyer 2003; Maaswinkel and Li 2003; Fleisch and Neuhauss 2006] or by the linear motion of a stripe pattern which may be placed under the animal [Neuhauss et al. 1999; Orger et al. 2000; Kroger et al. 2003; Orger and Baier 2005, Orger et. al, 2008]. The optomotor response is comprised of a routine turn followed by a rhythmic forward-swimming [Day, 2008]. These turns are generally oriented towards the direction of the stripe motion, roughly positioning the larvae at a perpendicular angle to the axis of stripe motion. The larvae are placed in a small chamber cut out of solidified agarose and restrained by a droplet of molten agarose, ventral surface down in a 35mm glass bottom Petri dish in order to observe behavioral response to OMR. The agar is removed from the “body” of the fish, to allow free movement of the tail. There should be no adverse effects from the anesthetic or the agarose embedding with regards to the observation of this behavior. The larva is placed (head-embedded) in a dish on a clear platform under the high-speed camera, which is mounted overhead, with some diffusion paper under the fish acting as a screen. The dish is illuminated using a fiber-optic light guide (Fiber-Lite, Dolan-Jenner, Lawrence MA). Each larva is allowed to acclimate to the arena for 5 minutes, with a stationary stripe pattern sitting underneath. A projector and small mirror at ~45 degrees are aligned so that gratings (pattern or blank and white stripes) are projected to the mirror can be seen on the screen under the fish. Grating timing, speed, and direction are all controlled from script written in LabView (National Instruments). An IR filter is inserted in 2 places in the light path: one in front of the light source beneath the fish, one in front of the camera before the light enters. To collect videos for OMR, the gratings and the video will be triggered simultaneously. During periods of relative quiescence, the stripe pattern is moved in a single direction at a uniform speed and the ensuing behaviors recorded. The width of the stripe will be 25 mm and the speed of stripe motion will be set for 1cm/sec at 5mm or 0.16 cycles/degree, as optimized for 5-day old fish [Haug et. al., 2010]. Recordings of either individual OMR movement bouts or a series of movement bouts will be saved to disk for analysis.

Rheotaxis: Larval zebrafish among other aquatic organisms are able to turn to face oncoming current. This behavior helps them to hold position in a flow rather than being swept downstream by the current. Even 5 dpf larval zebrafish has a lateral line system that has been shown to be responsive to water flow
[Metcalfe et al. 1985; McHenry and van Netten 2007]. Larvae (4-10 days old) for our behavioral battery will be placed in a flow channel (width = 1 cm) and connected to a pump that produced constant water flow. The flow apparatus is placed underneath a high-speed camera. When initially placed in the chamber, the larvae will be allowed to acclimate for 5-10 minutes without a current. An initial recording of swimming will be obtained after acclimation to the flow chamber and will be used as a baseline for the slow swimming. Then a steady current will be applied to the chamber and the resulting swimming behavior will be recorded for 1-5 seconds at 400 frames/sec.

Spontaneous Slow and Burst Swims: Swimming episodes of zebrafish larvae fall into two main types that are defined by different tail-beat frequencies (TBFs) and bend amplitudes [Budick and O’Malley, 2000] and also by differential use of their pectoral fins [Thorsen et. al, 2004]. While slow swims involve mild trunk bending at low (~30 Hz) TBFs, burst swims exhibit substantial yaw, high TBFs (which can be in excess of 80 Hz) and vigorous bending of the trunk. We will have observed these swimming patterns in the context of behaviors described above as Escape Behaviors include a burst swim component, while slow swim bouts are used during OMR and rheotaxis [Day, 2008]. But both kinds of swim bout can occur spontaneously and so when such spontaneous swim bouts are observed during our recording sessions, they will be saved for further analysis. Lesioned animals can exhibit a variety of abnormal swimming patterns and so the swim bouts observed in control fish (both spontaneous and elicited) will serve as comparison behaviors.

C.1.3 Quantitative Measurements

Kinematic analyses that best define the performance of each larval behavior will be performed. Listed below is a set of measurements that should serve this purpose, and without requiring excessive amounts of quantitation. As we progress with our lesion studies we may find particular kinematic features to be perturbed and so we may revise the measurements being made; since we are saving all of the raw behavioral recordings, we can always return to the control fish recordings to obtain additional measurements that are relevant for quantifying deficits in the lesioned animals. Our behavioral measures will be analyzed using NIH Image J, MatLab or by direct, manual measurements of the stored images. The measures below are comparable to those previously described for these zebrafish behaviors [Budick and O’Malley, 2000; Day, 2008].

Touch-Elicited Escape:
-- Escape Latency. This will be measured based on the time difference (msec) between the light tap to the head and the frame at which the first detectable escape movement is made.
-- Angular Velocity. This is calculated as the change in orientation of the larva during the initial turn (C-bend) divided by the duration of the turn.

Light-Evoked Swim:
--Tail Beat Frequency. The tail-beat cycle is the time required for one complete cycle of bending of the larvae’s right and left sides. TBF is 1/cycle time.
--Response Latency. The time from the onset of the increased illumination to the first detectable trunk movement.

Dark-Evoked Turn:
-- Angular Velocity. This is calculated as the change in orientation of the larva during a turn divided by the duration of the turn. Note that normal Dark-Evoked Turns have angular velocities that are far slower than the angular velocities of Escape Turns.
--Response Latency. The time from when the light is extinguished to the first detectable trunk movement.
**Optomotor Response:**
-- Offset Angle. This is the angle between the larva’s orientation and the axis of stripe motion—larger offset angles result in larger OMR turns. A larva that is perfectly aligned with the stripe motion axis would have an offset angle of 0°.
-- OMR Turn Angle. This is the change in orientation of the larval during its initial bend at the onset of the OMR response.
-- Tail Beat Frequency- The tail-beat cycle is the time required for one complete cycle of bending of the larvae’s right and left sides. TBF is 1/cycle time.

**Rheotaxis:**
-- Tail Beat Frequency- The tail-beat cycle is the time required for one complete cycle of bending of the larvae’s right and left sides. TBF is 1/cycle time.
-- Swim velocity will be recorded by dividing the distance traveled by the center of mass between each frame and the duration between the frames. The relative swim velocity will then be calculated by adding the flow speed of the water to the average swim velocity.

**C.2 Example Locomotor Behaviors**

**Touch-Elicited Escape Response**
Fast escape responses, such as the C-start escape behavior, are of central importance for zebrafish survival. The neural circuits that are responsible for the C-start escape response in the teleost fish has been investigated in great detail [Korn and Faber 1996; O’Malley et al. 1996; Liu and Fetcho 1999]. The Mauthner cells (M-Cell), are a large pair of reticulospinal cells, which receives sensory input directly at their dendrites and cross the midline of the body to extend the length of the spinal cord and initiate the C-start in the escape response. While the C-start has been shown to be elicited even when the M-cell is ablated [Zottoli and Freemer, 2003; Gahtan et. al., 2001; Eaton et. al., 2001] it has a longer latency [Liu and Fetcho, 1999; Bhatt et. al., 2004]. A study by Kohashi and Oda suggests that the firing of the M-cell is necessary for the fast escape response, whose latency is mediated by the acoustico-vestibular inputs [Kohashi and Oda, 2008]. Observations of larvae with proximal micropipette axotomy (described in the anatomical methods section below) are suggestive of a selective impairment of the second component of the escape behavior, the counter-turn. In some animals with large lesions we observed an asymmetrical counter-turn at 72 hours post axotomy in that the counter-turns to one side appeared normal (**Fig. 3**) whereas, after a head-tap on the opposite side of the larva, no counter-turn was observed (**Fig. 4**). The lesion in this animal included both Mauthner axons and a substantial number of other descending fibers (see **Fig. 8 below**). For this animal, two escape responses for both right and left sides were collected at 24 and 72 hours post injury and the asymmetry of the response persisted. Having observed similar kinds of deficits in other animals, we are investigating whether such asymmetries are due to the lesion or might instead reflect normal behavioral variability (Foreman and Eaton, 1994).
**Visuomotor Responses**

Visual stimuli have been shown to elicit motor responses, such as the optomotor response [Portugues and, Engert, 2009] prey tracking [McElligott and O’Malley, 2005] and navigational behaviors that include light-evoked swimming and dark-evoked turns [Day et al., 2006; Burgess and Granato, 2007]. Using IR illumination and a CCD camera, we observed similar navigational behaviors after both light off, which elicits a dark-evoked turn (Fig. 5) and light on, which elicits a gentle forward swimming (light-evoked swim) bout (Fig. 6). Though these preliminary examples were captured at slower frame rates, the locomotor battery will be collected at frame rates of 600-400 frames/second in order to ensure that we can detect subtle movement deficits following the experimental axotomies.

**Locomotor Battery**

We have also begun setting up an apparatus to collect the optomotor (OMR) response and have a swim channel with which we can measure rheotaxis. The rheotaxis, OMR, light- and dark-evoked and escape behaviors together encompass a range of sensory modalities and a range of neural systems that feed into the brainstem and spinal premotor systems. They also vary greatly in their motor outputs, from the extremely fast and powerful escape turns to the simpler and slower forward-swimming pattern seen after light-onset. Collectively, we expect that this set of behavioral observations will provide a fairly comprehensive and sensitive assessment of the larva’s overall locomotor capabilities.
Figure 5. Dark-Evoked Turn. This occurs in response to a sudden decrease of the field illumination, as shown for a 5-day old larva. Shortly after the light is extinguished, the larva begins to turn, making a complete directional change by the 4th frame, after which the larvae begins to swim forward and has stopped moving at 171ms. Images were captured at 64 frames/sec under infrared light.

Figure 6. Light-Evoked Swim. Larval zebrafish respond to increased illumination by either a forward swimming behavior or a routine turn followed by a forward swimming behavior, as seen in the example image here. This larva initiates its first movement at 359 msec following the presentation of the stimulus. Images were collected at 64 frames/sec.
C.3 Anatomical Lesions and the Regeneration of Injured Axons In Vivo

The potential to observe the regenerative capacity of varying sets of descending neurons has been previously demonstrated in the larval zebrafish [Bhatt et. al., 2004; Gahtan and O’Malley, 2001]. The transparency of the larval zebrafish allows us to also image these populations of cells in vivo using confocal and 2-photon microscopy. The experiments described below focus on determining the time-course and extent of anatomical regeneration. The two lesioning methods described here are complementary approaches to creating lesions of the brainstem and spinal circuits and provide varying degrees of control over the extent and locus of the lesion. In both cases, the goal is to observe the critical steps that occur in the regeneration of identified populations of axotomized, descending neurons.

Anatomical Methods

Distal Spinal Labeling
Larvae (4-5 days post fertilization) are anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (MS222; Sigma, St. Louis, MO) prior to an initial spinal injection of a 10% solution of 10,000 MW Alexa 488 dextran (Molecular Probes, Eugene, OR) to retrogradely label reticulospinal cell bodies in the hindbrain. The spinal injection procedure has been described previously [O’Malley and Fetcho, 2000; Gahtan and O’Malley, 2001]. A glass micropipette with tip broken to 15-20 µm was gently tapped into the spinal cord through the lateral axial muscle at approximately the level of the 23rd myotome (90% of body length) and a small amount (2.0nl - 4.0nl) of fluorescent tracer was pressure-ejected into the fish. Larvae are then placed in individual circular wells in 6-well tissue culture plates containing IO salt solution and placed in the incubator for 18 -24 hours. This period provides time for recovery and retrograde transport of the dye from the spinal axons to the somas of the reticulospinal neurons.

Proximal Micropipette Axotomy (Labeled Lesion)
Larvae are anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (MS222; Sigma, St. Louis, MO) following 18-24 hours after distal spinal labeling. A 50% w/v solution of 10,000 MW Texas Red dextran (Molecular Probes, Eugene, OR) was used to sever axons in the brainstem. Similar to spinal labeling, a glass micropipette with tip broken to 15-20 µm was lowered into the brainstem to cause a localized axonal disruption (Fig. 7), at approximately the level of the 3rd myotome a small amount (approximately 1.0nl -2.0nl) of fluorescent tracer is pressure-ejected [Gahtan & O’Malley, 2001] into the fish. This axotomy is referred to as a labeled lesion, since the injection of fluorescent dextran into rostral spinal cord simultaneously axotomizes descending neurons, and labels their cell bodies in brainstem as shown in Figure 7. Larvae are then returned to individual circular wells in 6-well tissue culture plates containing IO salt solution and placed in the incubator for another 18-24 hours. They are then placed in a small chamber cut out of solidified agarose and restrained by a droplet of molten agarose, dorsal surface down in a 35mm glass bottom Petri dish in preparation for confocal imaging. The anesthetic was removed and replaced with IO salt solution.

Figure 7: Retrograde labeling of descending neurons from far rostral spinal cord produces Labeled Lesions. Labeled lesion diagram showing the locus of injection into the spinal cord.
2-Photon Laser Scanning Microscope and Axotomy

A custom-built laser scanning microscope was used to image and perform the axotomy on the samples. In preparation the larvae were anesthetized and embedded in agarose as indicated in the micropipette axotomy. The detailed description of the imaging system can be found elsewhere [Warger et. al., 2007], but the relevant components are described here. The system is based on an inverted Nikon microscope with a fast polygonal-galvanometric laser scanning system. The polygon scanner consists of a mirror with 36 facets that spins at high speed and operates together with the galvo-based scanning system to scan a rectangular area on the sample. The galvo scanner can also be turned off so that the polygon scanner performs continuous line-scanning at a select location on the sample. In this configuration the line is scanned continuously across the sample. This line-scanning mode of scanning is required for the two-photon axotomy since laser power levels can be delivered continuously with this method. Also, there is less collateral damage with this method than scanning a large area with high laser power. The extent of the line was further limited by placing a mask or iris at one of the intermediate image planes. A two-photon image is captured to determine the desired site of axotomy. The region of interest is located on the two-photon microscope by imaging the sensory axons under low, non-damaging power. The iris is then closed down to reduce the size of the line to about 20 microns for the axotomy and a single line was scanned at a 6 kHz repetition rate across the axon.

A two-photon laser was used to image the samples and perform the axotomy. The laser consisted of a mode-locked Ti:sapphire pumped laser system (Tsunami) from Spectra Physics operating with 80 MHz pulse repetition rate. The pulses of the laser were about 100 fs in width and had about 12 nJ pulse energy at the peak output. The laser could be tuned from 700 nm to about 980 nm. This laser was used to image the sample by two-photon excitation and the filtered emission was detected by a photomultiplier tube (Hamamatsu, HC124-02). Typically, 20 mW average power (at the sample) was used to image the samples. After the samples were imaged, the axon was positioned and the scanner was switched to line-scanning mode and the laser intensity was increased to 30-50 mW average power (at the sample). It is important to note that the laser delivers much more energy to the sample in line-scanning than in imaging mode. In one second each pixel is visited 6000 times in line-scanning but only 10 times in 2D imaging mode and the axotomy is quicker.

A CCD camera was used to detect the emitted fluorescence during the axotomy and track the progress. The camera (Diagnostic Instruments, Inc., SPOT RT900) was cooled to -28 degrees Celsius and was used detect track the progress of the axotomy. When larger axons were cut it was possible to see an increase in fluorescence due to the apparent release of fluorophores into the surrounding area. This typically occurred after 3-5 min. of illumination of the larger axons with the laser. For smaller axons we saw loss of fluorescence signal when the axon was cut. This was probably due to the retraction of the two ends and removal of fluorophores from the focal region. Most of the imaging and axotomy was done using a 20X Plan Apo 0.75NA Nikon microscope objective. This objective had high throughput to the near-infrared laser and could resolve most of the fine axons. Additional Nikon microscope objectives such as a 60X or 100X Plan Apo 1.45NA are also available and might be used to image more details and cut smaller axons. These objectives, however, require more of the laser power since their near-infrared throughput is not good and have a shorter working distance (160 microns).

High-Resolution Confocal Imaging

A Zeiss Axiovert microscope with a 40 X, 0.75 NA objective and BioRad MRC600 laser scanning confocal microscope is also used for imaging reticulospinal neurons and descending axons in the intact larvae. Reticulospinal and other descending neurons were identified morphologically based on soma location and other anatomical features [Kimmel et al. 1985; Metcalfe et al. 1986].
Larval Health

Larvae are screened from each batch that we collect and only developmentally normal specimens are used in these experiments. For spinal injections the fish are minimally exposed (1-2 minutes maximum) to the anesthetic (MS222). The brief action of the MS222 is noted by the quick responsiveness of the larvae once they are placed back into the well-plates for recovery following spinal injections. The larvae are allowed a 24-hour recovery period before any assessment with the locomotor battery is performed in order to allow for recovery from non-neural damage. In instances where larvae are noted to be bent at any location following the spinal injection, they are discarded. Larvae that show only labeling of spinal interneurons or motoneurons (indicating likely axotomy), but that have no labeling (or apparent damage) of the descending neurons, serve as one of our control groups. These “spinal-neurons only” animals were previously seen to be behaviorally normal, presumably because there was only local, segmental damage and this was circumvented by the intact descending fibers. Locomotor battery testing on this group will further assess their behavioral normalcy, and if they seem normal, this will be a helpful control in that it rules out non-specific damage since these larval will have had penetration of the skin, muscle and the spinal cord in the same manner and degree as those larvae with axotomized descending neurons (where we will be looking for axotomy-specific damage).

C.4 Example Anatomical Results

Traumatic physical injury to the spinal cord leads to the mechanical disruption and degeneration of both ascending and descending axons. Of particular interest here are the connections between the descending neurons and their spinal targets, which are disrupted by axotomy. In regards to experimental axotomies, our primary interests lie in (1) observing the detailed changes that occur around the lesion site and (2) monitoring any regenerative responses of descending axons after both the proximal micropipette (labeled-lesion) axotomy and the 2-photon laser axotomy. For present purposes, we are using “axonal regeneration” to refer to the regrowth of axons after a severing axotomy. We aware that there may also be “sprouting” of processes from nearby, uninjured neurons, but our focus will be on axonal regeneration, since these are the fibers that we have labeled and that are being specifically axotomized.

Proximal Micropipette Axotomy (Labeled Lesion)

Preliminary anatomical results from our proximal micropipette axotomy indicates that we are (1) able to sever large subsets of descending axons, and (2) observe their structural responses for several days following the axotomy. Confocal imaging of fish from 24 hours post axotomy to 5 days post axotomy indicates that numerous retraction bulbs are best visible at 24 hours post lesion near the proximal injection site (Fig. 8A) and debris from severed axons including the Mauthner Axon (Fig. 8A) are being absorbed. At subsequent time points we are able to visualize large, more rounded retracted endings (Fig. 8B, C) as well as axon tips (Fig. 8C,D) that may be extending forward. The fish described here is the same specimen whose abnormal escape response was described in the Behavioral Results section.

Figure 8: Proximal Micropipette Axotomy. (A) Confocal image of severed descending neurons projecting into spinal cord, 24 hours post axotomy. The single bracket indicates the debris remaining from left Mauthner axon, whose large bulbous stump can be observed at the focus of the lesion (double brackets). At the focal area of the lesion several retraction bulbs are visible, even extending below the area of the lesion at 24 hours post axotomy. (B) At 5 days post axotomy, several retraction bulbs remain visible, including the left Mauthner, which has become more rounded in shape (arrow). Finer axons are also visible (insets C and D). (C) Top arrow indicates a large retraction bulb that was not traceable. The lower arrow is an extending axon tip most likely from Mi cells, as visible in B. (D) Larger retraction endings (thin arrows) are visible with fine axons running below them in this projection of a confocal image stack. Bead-like points, similar to nodes are visible along the length of the axon (wider arrows). Fish N133.
Figure 8.
2-Photon Laser Axotomy

Figures 10, 11 and 12 show a variety of responses seen at different times after cutting of axons with the pulsed laser on the Keck two-photon microscope. The cuts are believed to be due to 2-photon absorption processes occurring inside the fluorescent axon. Preliminary observations are shown below and we should note that we are working to obtain higher magnification images of the lesions site, so as to better observe and interpret the changes ensuing within the vicinity of the injury and also at other locations where regenerative responses of the axotomized neurons are taking place.

Preliminary Observations of Response to 2-Photon Axotomy

Descending axons that were previously labeled with Alexa 488 by an injection into the far caudal end of the spinal cord were cut using a 785 nm 2-photon custom-built laser scanning microscope. This technique allows us to create fine knife-like cuts, with potentially restricted damage. Our time-series data sets from this type of lesion indicate that we can observe (1) the length of time for the severed axons to retract in both the descending and ascending direction (Fig. 9), forming retraction bulbs, (2) identify smaller retraction bulbs and axon tips that may be regenerating, as well as (3) identify neuronal sprouting, as the surrounding tissue does not seem to have such a pronounced effect from the lesion. We are also able to monitor these fish using confocal microscopy over a period of 8 days following the regenerative response of the severed axons (Fig. 10). We can visualize axon tips of individual axons over the course of several days, which are descending towards the lesion and other axon tips that may be ascending. An interesting detail was also observed in this specimen where two axon tips were observed to meet and become entwined with one another (Fig. 11). Using the time-lapse images collected, we can then begin to reconstruct the severed axons and trace their paths (Fig. 12), along with uninjured axons. These results from both the micropipette axotomy and the 2-photon axotomy suggest that time-lapse imaging in the larval zebrafish should be a powerful tool for assessing the regenerative events of spinal cord injury.
**Figure 10.** Regenerative events following 2-photon axotomy (NP17) (A) An axon tip (arrow) is visible at 24hrs post axotomy. (B) Near the brainstem, at 48 hrs post axotomy, the same axon tip (seen in A) is extending forward (top arrow) via a long projection which has a small rounded ending. (C) Extending tip of another axon (arrow) that was located closer to the cell body is shown; two entangled axon endings are seen in the box and are shown at higher magnif. in Fig. 11. (D) At 8 days post axotomy, an axon tip (arrow) still remains visible.

**FIGURE 11:**

**Figure 11.** Entwined ends of two axons at 72-hours post axotomy (bracket) and an oval-shaped possible axon tip (NP17)

**FIGURE 12.**

**Figure 12.** Representative axons from Fig.10 for each time point (24 hrs, 48 hrs, 72 hrs, and 8 days) post- axotomy. This drawing is not to scale.
D. Research Plan

Overview: The central aims of this proposal are to (1) determine the extent of behavioral deficits produced by disruption of varying sets of nerve fibers descending into spinal cord and (2) to quantify the amount of anatomical regeneration and functional recovery that follows. After covering the individual Aims of this plan, we discuss how our observations can be combined to attain a better understanding of both the extent to which larval zebrafish can recover from spinal cord injuries and the potentially inherent limitations of this recovery. We begin by reviewing the elements of the locomotor battery (Aim #1) that will be used to quantify both the behavioral deficits produced by our lesioning techniques (Aim #2) and the locomotor recovery that is expected to follow (Aim #4). Locomotor recovery is expected to ensue from some combination of axonal regeneration and anatomical reorganization and (Aim #3) describes the approaches that we will use to quantify the neuroanatomical responses to the laser-cutting and axon-severing methods used in this proposal. While there are limits to what we can observe with our in vivo imaging techniques, our hope is that we can reveal sufficient details of the regenerative response in Aim #3 to understand the basis of the behavioral recovery that is quantified in Aim #4. Aim #5 (Nanotechnology Applications) is a distinct topic and is covered separately in the final section of this proposal; this Research Plan focuses instead on correlating the extent of spinal lesions with behavioral deficits, and correlating the extent of behavioral recovery with the regenerative response. This is elaborated upon in the concluding Discussion and Summary.

D.1 Locomotor Battery (Aim #1)

The first step in our research plan is to develop a fairly comprehensive locomotor battery that will allow us to quantify those behaviors normally produced by the larval Descending Motor Control System (DMCS). Though behavioral assays have been developed to systematically generate high-throughput environmental [Creton, 2009] or genetic screens [Orger et. al., 2004], the purpose of our locomotor battery is to allow us to rapidly and rigorously quantify the varying levels of locomotor deficits produced in larval zebrafish by our spinal axotomy experiments. As indicated in the Methods section, our locomotor battery will consist of mechanically and visually-elicited responses. The individual components of the battery are as follows; (1) touch-elicited escape, (2) light- and dark-evoked behaviors, (3) the optomotor response (OMR), (4) rheotaxis, (5) spontaneous slow and burst swims. In addition, we will record other spontaneous behaviors observed from both the control and the experimental larvae (which often exhibit a variety of abnormal behaviors; Day et al., 2005).

While this battery is not an all-encompassing catalogue of the range of behaviors displayed by these larvae (with prey-tracking behaviors being a deliberate omission), it satisfies the competing demands of efficiency and coverage of a range of locomotor patterns elicited by a variety of sensory stimuli. Our experimental time window is fixed within the 5-10 days post fertilization time frame as larvae are quite transparent across this time span and a large set of behaviors is well-established at the outset. Based on a number of studies the locomotor patterns observed are relatively stable throughout the day5 to day10 age range. Therefore, any observed locomotor recovery should reflect the regenerative capacity of the organism as opposed to an ongoing developmental process.

D.2 Locomotor Deficits produced by Labeled-Lesion and 2-Photon Axotomy (Aim #2)

Rationale for Lesioning Techniques

Using the labeled-lesion and the two-photon axotomy techniques, we will sever subsets of axons from the population of descending neurons. This will serve for establishing any ensuing locomotor deficits (Aim #2), observing any axonal regeneration that may subsequently occur (Aim #3) and quantifying the extent
of locomotor recovery that occurs (Aim #4). The size, location and extent of the anatomical lesions will be recorded using high-resolution in vivo confocal and 2-photon imaging. The two methods being employed can produce a diverse set of lesions, ranging from a single cut axon (disconnected neuron) up to (in theory) all of the ~300 neurons that make up the larval Descending Motor Control System. While moderate lesions (50-100 neurons) may produce only subtle or partial locomotor deficits in particular behaviors, the largest lesions (100-300 neurons) will provide the best opportunity to observe substantial locomotor deficits and their recovery. For labeled lesions, we will choose larvae with minimal to large lesions, i.e. varying in size from 5 to 300 neurons and including vary subsets of DMCS neurons. While small lesions might be expected to produce minimal to no deficits, this does depend on the particulars of the behavior being studied [see e.g. Liu and Fetcho, 1999; Gahtan et al, 2005; Orger et al, 2008]. The largest lesions are expected to produce the most severe deficits, in analogy with human patients with severe SCI and paralysis. Acquiring these ranges of lesions is thus important for our subsequent objectives of observing the degree of behavioral recovery and the extent of anatomical regeneration. In contrast, the 2-photon lesioning technique allows us to selectivly sever single, targeted axons, which offers some advantages for regeneration studies as discussed below.

Behavioral Testing

Based upon our observations thus far of larval recovery and on previously published results [Bhatt et. al., 2004], we expect to be able to observe the initial deficits produced by disconnecting axons from their spinal targets over a period of 1 to 2 days post axotomy. Beyond this time point, it is likely that functional recovery mechanisms will begin to manifest. This time window for assessing primary deficits may be modified based upon further behavioral testing, as needed. For each set of experimental animals, we will in general include several age-matched controls from the same clutch of eggs (i.e. raised in parallel) with the experimental animals to provide the best comparison groups for assessing the degree and types of deficits produced by both the Labeled-Lesion and 2-Photon axotomies. For each time point, each larva will be tested twice for each of the tasks in the locomotor battery. This in intended to provide a sufficient battery, while also avoiding undue stress and fatigue. In examining the behaviors of axotomized fish within the 24 to 48 hour window, we expect to find such disruptions of behaviors as decreased or altered responsiveness, abnormal bending patterns, and ineffective or unusual locomotor maneuvers. Prior work from our lab, at 24 hours post-lesion, has in fact shown highly unusual bending patterns, which was attributed to imbalanced and irregular descending control signals [Day et al., 2005]. In order to be efficient, we will first identify those fish that appear to show locomotor deficits as gauged by observing their responses to mechanical and visual stimuli. These same fish will be used in the subsequent aims observing anatomical regeneration (Aim #3) and locomotor recovery (Aim #4). Alternatively, fish with varying degrees of anatomical lesions might show relatively normal behaviors in some or all of the items in the locomotor battery. As noted in the Methods, we will quantify key parameters of each behavior to determine if they are in fact substantially similar to the control-animal measurements.

D.2.1 Complementary Approaches to the Axotomy of DMCS Axons

General Spinal Axotomy Plan

In order to observe the regeneration of severed axons within the spinal cord, this Aim will require injection of fluorescent tracer and spinal retrograde labeling from either a far-caudal location near the end of the larval spinal cord, at approximately the 23rd myotome (for subsequent 2-photon laser axotomy), or at a point near the juncture of brainstem and spinal cord (for Labeled Lesions). The labeled-lesion technique [Gahtan & O’Malley 2001], serves to retrogradely label populations of descending neurons whose axons can subsequently be visualized during the regenerative process. Batches of larvae that are 4-5 days old post-fertilization will be injected with 10,000MW Alexa 488, a stable and inert fluorescent dextran. The longevity and brightness of this dye had previously been established by the O’Malley lab for
Table 3. Instances of Laser Axotomy in the *C. elegans* and *Danio rerio* models

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<th>Model Organism</th>
<th>References</th>
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<td>Laser axotomy</td>
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<td><em>C. elegans</em></td>
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<td><em>Zebrafish</em></td>
<td>O’Brien et al. (2009)</td>
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**Two-Photon Axotomy vs. Labeled Lesions -- Figure 13**

Two-photon (2P) fluorescence microscopy has become a powerful tool for both observing neural circuitry and for *in vivo* optical surgery. This imaging mode allows for deeper tissue penetration compared to confocal microscopy, with better deep-tissue spatial resolution, and reduced photodamage. With both conventional fluorescence and confocal microscopy, the excitation light is increasingly absorbed with depth and contrast is also lost due to the scattering of both the incoming and emitted light. In comparison, the two-photon absorption process takes place in only a small volume at the focus of the laser beam and so it can provide three-dimensional resolution often at depths much deeper than what confocal can achieve [De Paola et al., 2006; O’Malley, 2008]. In the past few years, two-photon laser axotomy has been demonstrated in several animal models (Table 3) [O’Brien et. al., 2009; Hammarlund et. al., 2009; De-Miguel et. al., 2002]. In particular, in the nematode *C. elegans*, femtosecond pulsed lasers have been used to sever single axons, after which axonal regeneration can be observed [Yanik et al., 2004; Wu et al., 2007]. Following this approach, we have been using the 2-photon laser on the Keck Microscope, to cut either small bundles of axons or single-axons, in living zebrafish, depending on the amount of cellular labeling and the positioning of the cutting laser (Fig. 13). This approach also allows us to trace the cut axons back to the reticulospinal cell bodies in hindbrain, ideally permitting us to individually identify the neurons that have had their spinal axons severed. After the axotomy, the two-photon microscope can also be used in time-lapse mode to study the regenerative process (see below).

The labeled-lesion method is an alternative lesioning technique that entails inserting a sharp micropipette into rostral spinal cord and injecting fluorescent dextrans. This procedure simultaneously axotomizes sets of descending neurons and labels them sufficiently to identify their cell bodies in brainstem [Gahtan and O’Malley, 2001]. This technique is able to produce lesions that vary widely in terms of the numbers and identities of the descending neurons that have been axotomized, which can be useful in terms of producing a variety of locomotor deficits [Day et al., 2005]. Importantly, a single injection, which takes only a few minutes to make, can instantly axotomize up to several hundred descending neurons, after which the larva can be monitored for both axonal regeneration and behavioral recovery. Labeled-lesion experiments can also be viewed as *Neuronal Sparing Experiments* in that the non-labeled cells/axons are...
believed to be intact or spared. In cases where many descending neurons are labeled, we can infer that any spared locomotor behaviors are mediated by the remaining spared descending nerve fibers, to the extent that descending motor commands are required. This is of direct relevance to clinical efforts to restore locomotor function after SCI.

Both lesioning techniques have their respective strengths and limitations. With labeled-lesions one can make many lesioned fish and then use the confocal to screen for fish with labeling (lesion) patterns of interest. However, the injection penetrates the skin and muscle producing collateral damage and possibly a more pronounced immunological response that may hinder both our visualization of the lesion site and locomotor recovery (Fig. 13A). In comparison, 2-photon axotomy would seem to minimize collateral damage, ideally restricting damage to just the focal volume of 2-photon absorption (Fig. 13B). Two-photon microscopy may also enhance our ability to observe the behavior of severed axons both immediately after the injury and over a longer time frame, which is of importance for Aim #3. But care must be given, of course, to the dose of exciting light, as over-lasing can easily produce considerable collateral damage (unpublished data). It is also not clear, as yet, if we can generate the magnitude of lesions (i.e. numbers of axons severed) as easily or quickly with 2-photon axotomy as we can with the labeled-lesion method. The 2P technique can be viewed as a “virtual knife” that cuts in the middle of spinal cord, producing fine and restricted damage, but also potential allows us to sever large numbers of reticulospinal axons. At present, however, we do know that the labeled-lesion method allows us to create large substantial disconnections that produce specific locomotor deficits—whose recovery we will be able to quantify using our locomotor battery.

Because we will have both anatomical data on the lesion, and behavioral data on the deficit, we will be able to determine the extent to which specifics of the anatomical lesion are correlated to specifics of the behavioral deficit. Such details as size and location of the lesions may correlate with the severity and kinds of behavioral deficits. It also seems likely that specific details of the deficit may correlate with the particular individual neurons that have been axotomized [Liu and Fetcho, 1999; Gahtan et al, 2005; Orger et al, 2008]. The goal of Aim #2 is to record sufficient behavioral and anatomical details that we can extract the relevant correlations. While these efforts are aimed at understanding the severity of injuries and their recovery, this data may also shed light on more basic relationships between the identities of descending neurons and the production of specific locomotor patterns or behaviors.

Figure 13: Spinal Axotomy Methods (A) Confocal image of proximal-spinal micropipette axotomy (labeled lesion). The inserted glass micropipette is visible and points to the location of the axotomy. The axons coming from above are mainly reticulospinal axons, while those from below are spinal interneurons (including propriospinal neurons). Our focus is on descending axons; large retraction bulbs of both Mauthner neurons are visible here 24 hrs post-injury and are indicated by the traced axons at left (arrow). (B) Image of 2-photon laser axotomy at ~2 minutes post injury. The laser can be focused at any desired depth, therefore precisely localizing the focal volume of the axotomy. At this time point, we see that retraction bulbs have not yet formed, but the axons have begun to retract from one another, creating a small opening (arrow).
D.3 Visualization of Regenerating Axons Following Rostral Axotomy (Aim #3)

The experiments of Aim #3 will attempt to visualize the regeneration of axons severed by our two experimental lesioning techniques, as discussed in the preceding section. Essentially, Aim #3 entails taking larvae for which we have documented anatomical lesions and following them on our two-photon and confocal microscopes to monitor the initial response to the injury and subsequent regenerative events. Aside from Bhatt et al. (2004), we are not aware of other studies where the regeneration of individually-identified neurons has been followed in intact vertebrate animals, so this will be important for understanding the degree and extent of such axonal regeneration. We are also interested in specific details of the events occurring in and around the lesion site, whose visualization should be aided by our using our two complementary axotomy methods. Our hope is that these data will help to determine whether or not behavioral recovery (examined in the next aim, Aim #4) is a consequence of regeneration of severed descending axons as opposed to reorganizational or other regenerative processes.

Anatomical Data Analysis

When axons are completely severed, they are separated into two cytoplasmic segments (proximal and distal) with distinct fates. Other kinds of injury, such as nerve crush can result in related kinds of responses. Following an axotomy, the two cut ends of the axon typically are observed to retract, thereby leaving a gap in the axon. Our current observations indicate that the axoplasm begins to have a dark appearance, which might result from the breakdown of structural components of the cytoskeleton. There also may be swelling or shrinkage of axons as has been observed in the goldfish [OliPhint, 2010]. In order to observe these and other types of anatomical changes, we will acquire high-resolution images on the 2-photon and confocal microscopes. Imaging will primarily focus on the area where the axons were severed and adjacent areas of spinal cord where retraction bulbs, axonal swellings and sprouting nerve terminals may be observed. Whenever possible, we will attempt to trace the severed axons to their respective somas in order to determine the type of reticulospinal cells that were affected. As regeneration begins, we will acquire over several days anatomical records of the regeneration and attempt to trace and reconstruct the precise trajectories of the growing axon tips. This will allow us to quantify the extent of axon retraction and extension as a function of time. The axonal arbors we are attempting to reconstruct will generally be quite fine in comparison to the Mauthner axon, but our confocal microscope has optical zoom capabilities that will allow us to image axon tips at higher-resolution. We will also record other morphological details such as the shape and dynamics of the retraction bulbs and the regenerating axons.

Figure 14. Reconstruction of Axon Trajectories. Example of 3D reconstruction of severed axon bundles. This particular image shows axon morphology 24 hours post-axotomy. Freeware made available by Kristen Harris [Reconstruct, Harris, 1980; Harris and Teyler, 1983] will help us to visualize finer details of the regenerating axons.
With labeled-lesions we should, in principle, be able to see large numbers of regenerating axons. But to date such large-scale regeneration has not been visualized inside intact living fishes or mammals. This is true of our observations and also those reported by the Fetcho Laboratory, whose published work thus far is restricted to observations of just the regenerating Mauthner cells [Bhatt et al., 2004]. Nonetheless, both axotomy techniques will generate well-defined lesions, generally enabling us to identify the specific sets of reticulospinal neurons that have been disconnected from their targets in spinal cord. Our ability to image these axons in living animals, offers the prospect of following the injury response of single, identified axons over time, which may yield novel insights into those anatomical events occurring during the degenerative and regenerative processes. Such data will also be important for researchers focusing on molecular events occurring during degeneration and regeneration. For example, we see regenerating structures reminiscent of those observed in lamprey regeneration, which do not utilize growth cones like those seen during normal development [Jin et al., 2009]. Such differences lead to the impression that regeneration is a process that is fundamentally distinct from normal development.

D.4 Locomotor Recovery and Correlational Analysis (Aim #4)

Zebrafish larvae, axotomized as described above (Aim #2), will be monitored for behavioral recovery. Based on previous studies [Bhatt et al., 2004], we expect locomotor recovery may be largely complete at 5 days post-axotomy. However, if new data indicates that recovery takes longer, we can extend the behavioral observations accordingly. In observing the time course of locomotor recovery we will focus on those fish from Aim #2 exhibiting substantial deficits, as determined after the initial axotomies. Once again, at each recovery time point, each fish will be tested twice for each task, as defined in the locomotor battery. We will focus on those measures that best characterize the deficit, thus providing the best chance to see substantial locomotor improvement during the recovery phase. These larvae will be subjected to no more than 2 – 4 repeated attempts (on a given task on a given day), and we will also allow 10 minutes between trials when eliciting the targeted behaviors so as to avoid both post-injury fatigue and any performance-enhancing training effects.

We expect that those fish with large deficits will show considerable functional recovery over time. Note that fish with a unilateral axotomy will be tested for turning performance to both sides of the animal (i.e. the lesioned side and the intact side) in our evaluation of turn-encompassing behaviors (escape, OMR, rheotaxis, and routine turns). Detection of such behavioral asymmetries is a powerful assay since the intact side of the animal serves as an internal control [Liu and Fetcho, 1997; Orger et al., 2008]. We will perform quantitative analysis of kinematic details such as response latency, swim velocity, and tail-beat frequency over a period of time from 2 to 5 days post-lesion to compare with the initial deficits recorded. At each recovery time point we will also compare these locomotor parameters with those acquired from age-matched normal control fish. These measures are expected to be sensitive indicators of the degree of locomotor deficit and the subsequent behavioral recovery that may occur, which will presumably occur in parallel with neuroanatomical regeneration or reorganization.

Based on the observations from this data, we can not only correlate functional recovery with anatomical regenerative processes, but we will also learn how lesion size influences regenerative capacity and locomotor recovery. Just as there are varying degrees of human spinal cord injuries, the two techniques presented here offer a range of injury severity and thus provide a better means to determine the degree of locomotor recovery that this model organism is capable of. It should be noted that while rats and mice are routinely subjected to locomotor batteries to determine their recovery potential, no comprehensive locomotor battery has (to our knowledge) been applied to a lower vertebrate animal. As such, this proposal should generate fundamentally new and important data.
D.5 Discussion and Summary

In humans, the sudden loss of behavioral function following spinal cord injury (SCI) has two different functional states: an initial acute phase and a subsequent chronic condition. Current therapies are primarily aimed at minimizing the spread of damage during the acute phase and it appears that there is currently little that can be done in the chronic phase to promote meaningful recovery. Cervical SCI is a common and quite serious form of human SCI, often leaving patients with a paralyzing injury. However, the majority of SCI injury models describe observations obtained from injuries at the thoracic level. The model proposed here in the larval zebrafish presents an injury with some similarity to the human cervical injury problem. Each axotomy performed will allow us to observe the precise consequences of interrupting the flow of information from subsets of reticulospinal cells to their spinal targets. The largest axotomies will be useful in allowing us to create the greatest impairments and allow us to quantify the maximal degree of functional recovery. Minimal axotomies, on the other hand will provide a clearer visual window into the anatomical process of regeneration. By producing lesions that vary in numbers and identities of the disconnected neurons, we will be able to survey a broad range of behavioral and anatomical deficits and determine the degree to which anatomical and functional recovery vary. This determination should have significant implications for a broad range of clinical efforts attempting to restore descending motor control in mammalian models and humans.

The larval zebrafish offers several unique advantages to regeneration studies, stemming in part from its attributes as a model organism. There are a great variety of mutational, drug screening and genetic tools available for zebrafish and a large community interested in the value of zebrafish as disease and therapy models. At a more basic level, the simplicity and transparency of the larval CNS, particularly the precise identifiability of its spinal and brainstem neurons [Kimmel et al., 1982, 1985; Bernhardt et al., 1990], offers the potential to precisely define the normal connectivity from brainstem to spinal cord and the degree to which this connectivity is recapitulated in the regenerative process. While making this determination is beyond the scope of the present proposal, it seems certain that the fidelity and extent of regeneration will be determined in larval zebrafish before it is determined in any other vertebrate animal. What our proposed work will determine is the necessary foundation of baseline locomotor performance, the observed deficits and the degree of functional recovery. The correlations we make with the corresponding anatomical lesions, and the extent to which axonal regeneration occurs, further enhances the ability of this model to inform and interest pharmaceutical researchers and bioengineers who seek a test-bed to improve axonal and synaptic regeneration.

As a regeneration model, the larval zebrafish presents both opportunities and limitations. It is not a mammal, nor even an adult fish. While these remove it several steps from human SCI, it offers some offsetting benefits. Of great practical significance is the ability of the larval neurons to regenerate in a small number of days [Bhatt et. al., 2004, De Poister et. al., 2001], a process which can be observed in vivo, due to the transparency of the organism. This presents a huge advantage for researchers aiming to test or screen the benefits of molecular or engineering-based interventions and for the investigation of the molecular mechanisms of regeneration. This presupposes some relevance of larval regeneration to adult regeneration, and while we expect there will be substantial differences, there are at least some similarities. It is certainly the case that larval axonal “regeneration” is not simply a repeat of the earlier developmental process. This was made clear by Bhatt et al. [2004] who showed that, just as in adult animals, the Mauthner cell does not regenerate properly after axotomy. They also showed that cAMP enhances spinal regeneration, just as it does in adult lamprey [Jin et al., 2009]. Furthermore, our preliminary observation of the growing tips of regenerating reticulospinal axons is suggestive of a tubular profile looks similar to the regenerating structures seen in the adult lamprey. In neither lamprey nor zebrafish do we (as yet) have any evidence for involvement of growth cones during regeneration, despite their dominant role in the initial pathfinding of axons during actual development. While we certainly expect there will be substantial differences between regenerating larval zebrafish axons and regenerating adult rat...
E. Estimated Time-Table for Completion of Dissertation

**Late Summer 2010:**
Submission of final proposal to committee
Proposal defense

**Early Fall 2010:**
Complete Aim 1
Continue data collection on Aim 3

**Late Fall 2010:**
Begin work on Aims 2 & 4
Continue data collection on Aim 3

**Spring 2011:**
Continue data collection and analysis for Aims 2 - 4
Meet with committee to assess progress and obtain feedback
Begin manuscript process

**Fall 2011:**
Complete last experiments and data analysis
Write and defend thesis
F. Pilot Experiments in Nanotechnology

Steps Towards Developing New Therapies for Spinal Cord Injuries

Overview: The use of nanotechnology in various areas of the biological sciences and medicine could revolutionize the methods by which we conduct scientific experiments and apply our understanding to treating human disease states. Long-term, non-invasive techniques using nanotechnology are currently being tested for its efficacy in regenerative medicine. The experiments described here contribute to Aim #5, which employs both nanoparticles and nanowires in efforts to develop novel therapeutic approaches. The details described regarding these two projects were a result of the work completed under the IGERT Nanomedicine Science and Technology fellowship, created with support from the National Cancer Institute and the National Science Foundation.

The goals of the two nanotechnology-related projects were quite different, though their ultimate purpose of applying these concepts to the practical idea of treating individuals with spinal cord injuries was singular. The delivery of nanoparticle to the cell body would establish an important technical paradigm since the retrograde transport of functionalized nanoparticles has not previously been documented. If successful, the future goal would be to test the effects of candidate repair genes in a system in which both the regenerating descending neurons and target spinal neurons can be individually identified and visualized inside the intact, regenerating animal. The development of a viable nanowire interface would allow us in future to directly test the efficacy of nanoscale brain-machine interfaces inside a vertebrate animal. We performed validation experiments using cultured cell lines help to establish effective nanowire array dimensions for future recording and stimulation of neural activity. The conjunction of these two approaches presents steps towards the development of varying opportunities that can enhance not only the treatment of spinal cord injury, but also a much broader variety of neurological and neurodegenerative disorders.

F.1 Nanotechnology Methods

Fluoro-Nanogold Spinal Injection

Larvae were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (MS222; Sigma, St. Louis, MO) following 18-24 hours after spinal labeling. A solution of Fluoronanogold (FluoroProbes) was injected to observe if these 10nm nanoparticles were capable of retrograde transport to the cell bodies of severed axons. Similar to spinal labeling, a glass micropipette with tip broken to 15-20 mm was lowered into the brainstem to cause a localized axonal disruption, at approximately the level of the 5th myotome a small amount (1.0nl -2.0nl) of fluorescent tracer was pressure-ejected into the fish. Larvae were then returned to individual circular wells in 6-well tissue culture plates containing IO salt solution and placed in the incubator for another 18-24hours.

Fabrication of Gold (Au) Nanowires

Electrochemical anodization and electrodeposition provides a feasible means by which to produce an array of gold nanowires inside a nanoporous alumina template. Therefore, we first prepared nanoporous alumina templates with controlled pore diameter and length which was attained by anodizing commercially available 99.995% pure aluminum foil in electrolytic solutions, such as 15% sulfuric acid, 3% oxalic acid, 5% phosphoric acid, etc. The anodization was carried out under DC conditions by placing an aluminum foil and a platinum mesh as the anode and cathode respectively (Figure ). Maintaining a separation of 10cm produced a uniform electric field at the Al.

Smaller pore diameters of 10nm were produced by anodizing Al
foil with 15% sulfuric acid at 10V. To create templates with larger diameters of 40nm and 80nm, foil was anodized in 3% oxalic acid at 40V and 10% oxalic acid at 60V respectively (Figure 1). Consistent results of 10nm pores were obtained by anodization with 15% sulfuric acid at 10V.

**Figure 2:** A) Schematic of nanoporous alumina template structure [4, 5]. B, d: Determined by Anodization Voltage and Acid; alumina layer thickness (B ~10-20 nm, d ~ 10-150 nm). L: Determined by Anodization Time; height of the nanowire (~ several mm). D: interpore separation; B) Changing pore length as a variable of time during anodization.

**Figure 3:** SEM images showing pore patterns in nanoporous alumina. (A) Cross-section image showing vertically arranged pores with diameter 40nm prepared by anodization in 3% oxalic acid at 40V and (B) top view image of a template prepared by anodization in 5% phosphoric acid with pores diameter of 75nm.

To synthesize the Au wires within the template, electrodeposition was performed by using an electrolyte containing HAuCl4+4H2O (0.93 g/l) and boric acid, H3BO3 (30 g/l) with pH 1.5. Under AC conditions at 250Hz, 12V electrodeposition was achieved by controlled accumulation of Au3+ ions, which combine with 3 electrons during every negative half cycle. The length of Au nanowires described in this chemical reaction is determined by the electrodeposition conditions (time of deposition and AC frequency). The diameter of the wires reflects the original pore diameter of the template. Therefore, this method renders precisely controlled conditions allowing us to consistently replicate each Au nanowire array (Figure 4).

Next follows a series of steps to etch out the nanowires from both sides of the template. This begins by chemical etching and ion milling of the top surface of the aluminum oxide. The process begins with a chemical etching of the sample with a dilute (5%) phosphoric acid and is followed by ion milling to create a uniform length nanowire surface close to the template. The etched sample is then covered with a protective organic layer followed by removal of the aluminum layer in mercuric chloride solution. This is followed by etching of the thin barrier layer of aluminum oxide (∼ 10-40 nm) below the pores with dilute (5%) phosphoric acid solution. In the final step of this process, the protective organic layer is removed in alcohol. The resulting sample then consists of a protruding array of Au nanowires from both surfaces of the template where the midsection of the nanowires are firmly embedded inside the template. To then

**Figure 4:** Integration of Nanowires with Electrodes. A) Schematic diagram showing the top and side view of the nanowire array device, B) Microelectrode array fabricated using E-beam Lithography, C) Wire-Bonding to Electrodes
integrate this nanowire array with an Au electrode array we begin by uniformly coating a thin layer of negative resist polymethylmethacrylate (PMMA) \(\sim 150\) nm at the surface of the template and baking at 180°C for 90 seconds for curing. Using CAD (computer-aided design) an appropriate resolution design was then patterned onto the PMMA in the scanning electron microscope (SEM) by E-beam lithography (EBL). The pattern was developed and the exposed region was removed in methyl-iso-butyl-ketone and iso-propyl alcohol solution. To assure specific Au adhesion a final plasma etch is performed to remove any further traces of PMMA. The sample is then sputtered with gold and followed by a four day lift-off in acetone leaving Au microelectrodes attached to nanowires. The wire bonded Au wire can then be attached to macroscopic wires in order to connect to external devices for recording and stimulation of neuronal cells (Figure).

**Neuroblastoma Cell Cultures**

As a means to evaluate the performance of Au nanowire array we began by culturing Mouse neuroblastoma cells (N1E-115) purchased from ATCC on the opposite side of the EBL contact pads. The cells were cultured in flasks containing 90% Dulbecco’s modified Eagles’ medium with 4.5g/L glucose (without sodium pyruvate) (DMEM; Invitrogen), 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin, and 100 µg/ml streptomycin and maintained at 37°C with 5% CO\(_2\) in a humidified atmosphere and passed every 3-4 days. These cells are a clone from the mouse neuroblastoma line C-1300 and retain many of the properties of differentiated neuronal cells. In order to create differentiated cultures, the cells were first plated on 30mm plastic dishes with 20mm glass inserts that were coated with 0.1% W/V poly-L-Lysine (Sigma) or 2.5 µg/ml ECL matrix (entactin-collagen-IV-laminin) (Upstate Cell Signaling Solutions) and incubated overnight at 37°C. The cells were then plated in differentiation medium (96% DMEM supplemented with 2.5% of FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1.5% of DMSO) or serum starved. Medium was changed every 48hrs.

**Hippocampal Cell Cultures**

In order to better understand the patterning of synaptic connectivity between neurons at the level of individual synapses our study has now come to focus on the properties of cultured hippocampal networks. Therefore, neuronal hippocampal cultures were prepared from E18 Sprague-Dawley rats acquired from BrainBits (Springfield, IL). Dissociation of cells was performed by triturating the tissue through a fire-constricted Pasteur pipette and spun at 11000rpm for 1 minute. The supernatant was then discarded and the pellet was resuspended in B27/Neurobasal + 0.5mM glutamine without glutamate (Invitrogen) and plated onto enactin-collagen-IV-laminin (Upstate Cell Signaling Solutions) coated coverslips (12mm diameter) as described previously. The cultures were untouched for 3 days and then one half the media was changed every three days following. The cells were grown in a 37°C with 5% CO\(_2\) and 20% O\(_2\) in a humidified atmosphere.

**F.2 Results of Pilot Experiments**

**Retrograde Delivery of Nanoparticles**

Functionalized nanoparticles have recently been demonstrated to be efficient systems for delivering DNA into cells (Kommareddy et al., 2005). The retrograde transport of such particles, from the site of damaged axons in spinal cord, back to the healthy cell bodies in brainstem, therefore offers a potential means of delivering therapeutic compounds, including DNA and RNA, in an effort to promote axonal and synaptic regeneration. However, there have been few successful demonstrations of retrograde transport of a plasmid or other exogenous gene, outside of the well known viral tracers such as pseudorabies virus. Importantly, there has been no demonstration of selective and efficient transport of DNA or RNA specifically into damaged axons so that they might specifically stimulate (in the case of spinal cord injury) the descending neurons whose axons need to be regenerated.

Retrograde delivery of nanoparticles seeks to utilize natural axonal transport mechanisms for the purpose of transporting therapeutic nanoparticles to the neuronal cell bodies of the damaged neurons [Patel et. al., 2009]. Figure shows a typical experiment in larval zebrafish where neurons are simultaneously labeled.
and damaged (axotomized) in rostral spinal cord to simulate a high-cervical spinal cord injury, effectively paralyzing the larval zebrafish. In developing this “labeled-lesion” method we showed that after severing reticulospinal axons, large dextrans could diffuse into the patent opening and then be trapped due to rescaling of the axon segment that remained connected to the cell body [Gahtan & O’Malley 2001, Gahtan & O’Malley, 2003]. The left panel (red cell bodies and axons) shows typical retrograde labeling of brainstem cell bodies that have taken up a retrograde tracer, Texas red dextran-10,000MW, which was injected into the rostral spinal cord (Figure). The right panel shows labeling of cell bodies (double arrowhead) and axons (arrows) in spinal cord that were labeled by injection of a commercially available nanoparticle, Alexa 488-Fluoro-Nanogold-Streptavidin (FNS) (Nanoprobes Inc.). Earlier attempts to transport FITC or EGFP labeled nanogold particles had not worked apparently because of solubility problems, but the FNS proved easy to work with and thus offers a potential means to deliver therapeutic genes to defined populations of descending neurons – and specifically to neurons damaged in spinal cord injury. While the strepavidin domain offers a means to deliver therapeutic genes, the gold and fluorophore moieties allow for effective visualization of such transport.

Figure 5: Confocal imaging (BioRad MRC600) of 5 day larval zebrafish hindbrain. A) Retrograde labeling is seen as indicated by the red axons (single arrowhead) and cell bodies (double arrowhead) (Texas Red dextran) where a subset of reticulospinal neurons has been labeled. B) Similar injection performed with Fluoro NanoGold, which is seen to also have potential retrograde transport properties in the larval zebrafish as it is noted to label axons (single arrowhead) and cell bodies (double arrowhead) as it travels towards the hindbrain.

In preliminary experiments we injected mixtures of Texas-red dextran and FNS nanoparticle into larval zebrafish spinal cord and typically examined larvae the next day for evidence of transport. Figure shows three panels of the same stretch of spinal cord in which both Texas-red dextran and FNS have been transported rostrally from the injection site (below the image) towards the brain (above the image). This experiment took advantage of the Keck 3D fusion microscope which allows us to view the same field using a variety of imaging modes [Warger et. al, 2007]. In this case fluorescence confocal imaging (left, middle) revealed an extended structure with bulbous expansions (possibly a reactive damaged axon). The Texas-red and Alexa 488 labels show a similar distribution indicating good mixing of the highly soluble dextrans with the FNS particles. These patterns also mapped well onto our direct visualization of the nanogold moiety of FNS, which we visualized using two-photon microscopy (right). The overall pattern of visualization was essentially identical across the 3 labels.
Development of Reliable Biocompatible Surface on Nanowires

The interface between biology and technology has been widely explored in the last several decades. The existing field of electrophysiology for example explores electrical properties of biological tissues. The leap from tissue to cells is fundamentally a problem of resolution in the sense that the devices employed must be able to record from smaller regions while still maintaining sufficient signal coherence. Similarly, the leap from many cells to an individual cell faces the same difficulty. To overcome the problem of resolution, nanotechnology is being used to fabricate prototype sensors [Wang et. al.,2006, Patolsky et. al.,2006].

Linas et al. demonstrated the use of 0.6µm Pt electrodes to obtain electrical recordings from the spinal cord via its vascular capillary bed, thus demonstrating that the intravascular space may be utilized as a means to address brain activity. Recently, microchip nanoelectrode arrays based on carbon nanotubes (CNT) have been fabricated by Nguyen-Vu et. al. In their work, the microchip consists of vertically aligned CNT arrays (both insulated and uninsulated) on multiple individually addressed microelectrode pads. Such CNT nanoelectrodes can be potentially useful for measuring simultaneously extracellular neurotransmitters and electrical activity. However, CNT nanoelectrodes present several issues related to biocompatibility and toxicity which can make them unsuitable for in vivo studies. Patolsky et al., have also reported the use of silicon nanowires to detect and stimulate nerve signals along axons and dendrites of neurons. In their work, the electrode configuration is horizontal rather than vertical. Their chip consisted of 20nm wide silicon wires running across its surface.

The nanowires that were used in this work to investigate nano-neuro interactions is quite distinct with potentially greater capabilities than the configurations studied by Nguyen-Vu et al. and Patolsky et al. We have used use Au nanowires which are highly conducting, biocompatible and non-
toxic (unlike the toxic CNT’s). They are arranged vertically (unlike the horizontal array in Patolsky’s work) in the form of a rectangular grid which would allow simultaneous recording from several different sites on the neuron, in future. A schematic diagram showing our concept is shown in Figure 7.

The nano-bio device that we produced consists of a vertical array of conducting gold nanowires (10nm), which were prepared by means of electro-deposition (Figure ) inside nanoporous alumina templates and then integrated with microscale electrodes at one end. The length of Au nanowires described in this chemical reaction is determined by the electrodeposition conditions. The diameter of the wires reflects the original pore diameter of the template. Therefore, this method renders precisely controlled conditions allowing us to consistently replicate each Au nanowire array. These nanowire arrays (Figure 8) can potentially then be directly interfaced with cultured primary neuronal cells. The challenging aspect in such a device is the integration of each of the nano/micro electrodes to macroscopic wires. In order to wire-bond the electrodes with an external device, at least one dimension of the electrode must be of the order of 100μm which is considerably large compared to the surface area of the device. This effectively reduces the number of electrodes that can be contained in the final device.

In order to create an effective closed-loop control system, we have begun to study the electrical resistivity of the gold nanowires. An estimate of the nanowire-array resistance is also important in order to decide what parameters (electrode dimensions, applied voltages/currents) are suitable for neuronal recordings. We used a two-probe technique to measure the resistance of templates containing gold nanowires. These measurements were taken by attaching macroscale gold wires to the upper and lower surface of the templates, across which dc voltage was applied. Figure 9 shows measurements made from 10 nm gold
nanowire under dry conditions that show increasing voltage with an increase in current. These measurements should be repeated under varying conditions for studying neuronal cultures. In future, our measured values for the resistivity of the nanowires will provide appropriate values of the input voltage/current for optimal recordings.

Finally our results in the fabrication of such a device and the interaction of such a nanowire-array with primary rat hippocampal cultures are described briefly. One of the major problems with long-term neural recording is the motion of the cell. Therefore in order to create a tight interface between the nanowires and the cell membrane, we have tested cell adhesion molecules (CAMs) that will enhance the stability, electronic coupling and mode of interaction between the device and the cells. Proteins (CAMs) are located on the cell surface and are involved with the binding with other cells or with the extracellular matrix (ECM) in the process called cell adhesion. Enactin-Collagen IV-Laminin (ECL) cell attachment matrix from Engelbreth-Holm-Swarm (EHS) mouse tumor (Millipore), and laminin from EHS murine sarcoma basement membrane (Sigma) were used to study the attachment properties of primary E18 hippocampal cells on four surfaces: glass coverslips, glass coverslips coated with a thin film of gold, aluminum oxide membranes (without nanowires) and our gold nanowire templates. Each of these surfaces was coated with ECL or laminin and the neurons were cultured for two weeks and visualized using light microscopy and scanning electron microscopy. Our studies showed that while ECL is an excellent matrix for cell attachment, laminin is found to promote better cell outgrowth [Gelain et. al., 2007] and survival at similar time-points on glass coverslips and the nanowire arrays (Figure 10). Additionally, other improvements such as argon plasma cleaning of the templates prior to cell culture under high pressure have also been found to be promising to create a healthier interaction between the cells and the nanowire template. Figure 11 shows scanning electron microscopy images of healthy hippocampal neural cell cultures on standard cell culture plates, thin gold films and nanowire arrays consisting of 10nm gold nanowires in aluminum oxide templates. Considering the many challenges, we have at this juncture been able to demonstrate a basic biocompatible nanowire array that will provide a platform for culturing sustainable primary neurons, which may be used to study the interaction of neuronal networks. Further experiments will be necessary to evaluate the influence of wire array parameters (wire size, height and spacing/density) on these morphological measures.

Figure 10: Cell attachment matrix ECL & Laminin 1) Growth of primary rat hippocampal cultures on coverslips shows elongating axons (black arrows) and cells adherent to the ECL matrix (1A, 1B). 1A) 9 day, 1B) 14 day 1C) Slower cellular growth on aluminum oxide membranes (Whatman Anodisc without Au nanowires) at 14 days, demonstrating porous alumina clearly supports the growth of primary neurons. 2) Cell attachment factor Laminin, Growth of primary rat hippocampal cultures on glass coated coverslips (2A,2B) shows a similar pattern of growth to the ECL with vigorous elongating axons (black arrows). 2A) 9 days, 2B) 14 days, 2C) Healthy cellular growth on aluminum oxide membrane at 14 days is also visible, though the opaque surface makes it more difficult to identify elongating axons. Scale Bar = 50µm.
Figure 11: Neuronal Morphology as Visualized with SEM
A) Primary culture morphology of E18 hippocampal neurons at 5 days on glass coverslips. B) 18 day old cultures of hippocampal cells near the edge of the gold nanowire array. C) Morphology of attached cells on the gold nanowire array surface. D) Intertwined axons on array surface of hippocampal neurons from C.
G. Bibliography


