Video Article

Motor Nerve Transection and Time-lapse Imaging of Glial Cell Behaviors in Live Zebrafish

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Abstract

The nervous system is often described as a hard-wired component of the body even though it is a considerably fluid organ system that reacts to external stimuli in a consistent, stereotyped manner, while maintaining incredible flexibility and plasticity. Unlike the central nervous system (CNS), the peripheral nervous system (PNS) is capable of significant repair, but we have only just begun to understand the cellular and molecular mechanisms that govern this phenomenon. Using zebrafish as a model system, we have the unprecedented opportunity to couple regenerative studies with *in vivo* imaging and genetic manipulation. Peripheral nerves are composed of axons surrounded by layers of glia and connective tissue. Axons are ensheathed by myelinating or non-myelinating Schwann cells, which are in turn wrapped into a fascicle by a cellular sheath called the perineurium. Following an injury, adult peripheral nerves have the remarkable capacity to remove damaged axonal debris and re-innervate targets. To investigate the roles of all peripheral glia in PNS regeneration, we describe here an axon transection assay that uses a commercially available nitrogen-pumped dye laser to axotomize motor nerves in live transgenic zebrafish. We further describe the methods to couple these experiments to time-lapse imaging of injured and control nerves. This experimental paradigm can be used to not only assess the role that glia play in nerve regeneration, but can also be the platform for elucidating the molecular mechanisms that govern nervous system repair.

Video Link

The video component of this article can be found at http://www.jove.com/video/50621/

Introduction

Zebrafish have been used extensively to study development of the nervous system because of their optical transparence and ease of transgenesis, which when coupled, allow for spectacular imaging of dynamic cell behaviors in a living embryo. Additionally, because zebrafish and mammals share nearly all of the genes required for nervous system formation, cellular and molecular information collected in this model organism is directly relatable to other vertebrate species. Although incredibly powerful for neural developmental studies, the zebrafish and its unique attributes have the potential to also elucidate the mechanisms that maintain and rebuild the nervous system after injury. Zebrafish larvae maintain their translucence into late larval stages and pigmentation can be effectively blocked with either the use of pharmacological inhibitors of melanin production or genetic mutants that lack pigment cells. Thus, using this model organism to study injury and regeneration in older animals is possible and offers the unique opportunity to directly investigate the cellular and molecular mechanisms that rebuild the nervous system. In this manuscript, we describe how to efficiently and reproducibly injure nerves in the PNS of zebrafish larvae. This injury paradigm lends itself to studying not only degeneration, but also the responses of peripheral glia and immune cells as well as the interactions between these populations during regeneration.

The PNS is a complex network of motor and sensory nerves that is necessary to pass information between the central nervous system (CNS) and the skin, organs and muscle of the body, allowing an organism to interact with its environment and survive. Along these nerves, peripheral glia, including myelinating and non-myelinating Schwann cells and perineurial glia, as well as connective tissue, encase the axons and ultimately form the mature nerve. Injury of these nerves initiates a process known as Wallerian degeneration [10]. This mechanism of axonal fragmentation, immune recruitment, debris clearance and regeneration is very stereotyped and genetically regulated [1]. Previous studies in mammalian systems have described the roles of Schwann cells during nerve degeneration and regeneration [1, 2, 6, 8]. In these studies of fixed tissue or cell culture, Schwann cells not only recruited macrophages to the injury site to aid in debris clearance, but also aided in myelin phagocytosis themselves. While these studies have been incredibly informative, we have never before visualized glial responses to peripheral axon injury *in vivo* in real time, and no other studies have investigated the relationship between the different classes of peripheral glia during these events.

Recently, several labs have investigated Wallerian degeneration using zebrafish and laser-mediated axon injury similar to what we describe here [4, 5, 7, 9]. In some of these studies, superficial sensory axons were axotomized in young larvae using a custom built, two-photon confocal microscope [4, 5, 9]. In another study, which is very similar to our own, deeper axons within the ventral motor nerve were transected in 5 day old

larvae using a commercially available laser ablation system [7]. In both of these experimental set-ups, the focus was on Wallerian degeneration and both axons and immune cells were imaged. To expand on these studies, we describe injuring motor axons in older larvae with more mature, myelinated nerves and assay the response of all nerve-associated peripheral glia during degeneration and regeneration.

To do this, we transect motor nerves in 6 and 7 day post fertilization (dpf) larvae and visualize the responses of individual glial populations as well as investigate the interactions between these populations along injured axons. Using double and triple transgenic lines that label peripheral glia, including Schwann cells and perineurial glia, as well as a marker for axons, we use a commercially available laser ablation system consisting of a nitrogen-pumped dye laser (wavelength 435 nm) attached to a spinning disc confocal system to create axon transections. This experimental set-up allows us to visualize live, larval zebrafish, injure specific peripheral motor axon tracts and time-lapse image the responses of distinct glial populations to axon injury and their relationship to one another. This protocol can be further adapted to create nerve injuries in zebrafish of different ages, with different transgenic lines or genetic mutants to address different scientific questions.

Protocol

1. Preparation and Mounting of Zebrafish Embryos for Ablation and Live Imaging

1. Prepare a stock of 0.8% low melt agarose in egg water. Aliquot into 13X 100 mm disposable culture tubes and store at 4 °C until needed.
2. Cross adult zebrafish containing stably integrated transgenes to fluorescently label motor neurons and glial cell types of interest. Collect zebrafish embryos in egg water and place in 28.5 °C incubator for correct staging later [3].
3. At approximately 24 hr post fertilization (hpf), remove egg water and add 0.002% 1-phenyl 2-thiourea (PTU) in egg water. Return embryos to the incubator.
4. Between 24 and 96 hpf, embryos should be screened for the presence of desired transgenes on a fluorescent dissecting scope. Place selected embryos in fresh PTU egg water and return to the incubator.
5. When larvae have reached 6 days post fertilization (dpf) (or desired age), remove them from the incubator, select a few larvae for mounting, and transfer them to a smaller dish.
6. Remove the water from the dish and immediately replace with approximately 0.02% Tricane in PTU egg water. Allow larvae to sit in anesthetic approximately 5 min.
7. Place an aliquot of 0.8% low melt agarose in a beaker with tap water and microwave for 30 sec, or until the agarose is melted. Allow the beaker to cool until the agarose in the culture tube feels lukewarm to the touch (not hot).
8. Select an anesthetized larva and transfer it to a single or multi-well 35 mm glass bottom dish. Remove any water that was transferred with the larva, then immediately cover the larva with enough warm agarose to fill the glass-bottomed portion of the dish but not create a large dome.
9. As the agarose hardens, use a dissecting needle to position the larva on its side at the bottom of the dish, then tilt the larva just slightly on its back. It is imperative for injury and subsequent imaging, that the mounted larva be touching the glass on the bottom of the dish. Use the dissecting needle to maintain the larva in this position until the agarose is solidified and the larva is immobilized.
10. Once the agarose is fully hardened, slowly pipette enough Tricane water (this can be the same Tricane used for anesthesia) into the dish to completely cover the agarose and larva.

2. Laser Calibration and Testing

1. Turn on all confocal microscope instrumentation, appropriate diode lasers for exciting zebrafish transgenes, and the nitrogen-pumped dye laser. Be sure the "blank" beam splitter is in place, the laser attenuator is fully open, and the 435 nm Coumarin dye cell is in place. On the computer, open imaging software.
2. Move a 63x 1.2NA water immersion objective into position and apply a small drop of immersion medium for water objectives onto the objective. A 63x objective will allow for good laser ablation accuracy, and water immersion allows for a larger working distance, which is necessary when working with 6-7 dpf zebrafish larvae.
3. Obtain a glass slide with one mirrored side to use for calibration of the laser. Place the slide, mirror side down, onto the microscope stage.
4. Using the eyepiece under brightfield illumination, find and focus on a scratch or etch in the mirror. The brightfield light will be shining down onto the glass slide, but will only pass through to the objective in places where the mirror has been scratched or etched away, causing the mirror to look black through the eyepiece, and the etchings to look like spots or lines of light.
5. View and focus the etchings on the computer screen using imaging software. Open the window that controls the laser calibration and power settings. Set the number of pulses to "1" and the attenuation plate to "3" % transmission. The number of pulses represents the number of times the laser will fire within each region of interest (ROI) and the % transmission represents the laser power. Be sure the correct calibration setting is selected for the 63x 1.2NA water immersion objective.
6. Select the ellipse tool from the main toolbar, and click the image on the computer screen to create a single circular ROI. Do this 3 more times until there are 4 circular ROIs spaced randomly over the image.
7. Some systems may include a safety feature that will not allow the laser to fire if the light path to the oculars is open. If this is the case, manually close the light path to the oculars at this time.
8. Fire the laser. This will create 4 small spot etchings, 1 within each circular ROI. If the laser does not fire, check to be sure the laser is turned on and connected properly, and that the light path to the oculars is closed. If the laser fires but no etching is seen, check to be sure the "blank" beam splitter is in place. If everything is in place, increase the laser power and "frap" until the etchings are visible. If a laser power setting greater than 10 is necessary to etch the glass, this may indicate the laser plasma cartridge needs to be replaced.
9. If the etched spots appear centered within the selected ROIs, no calibration is necessary (proceed to 2.12). If the spots are not centered, the calibration setting needs to be updated.
10. To calibrate, click the update the calibration setting. The laser will fire and an image will appear containing a single etched point. If the point does not appear, cancel the calibration, increase the laser power, and start the calibration again.
11. Click in the center of the spot. The laser will fire again, and another point will appear. Click that point, and repeat this process until the calibration is complete and 9 spots appear in a grid fashion. Repeat steps 2.6-2.9 to check that the calibration was successful.

12. Remove the glass slide from the microscope stage and clean the objective. Open the light path to the oculars, and replace the "blank" beam splitter with the "100%ILL" beam splitter.

3. Nerve Transection Using Laser Ablation and Time-lapse Confocal Imaging of Glial Cell Behaviors

1. Remove the stage used to hold the glass slide and replace with a stage suitable for holding 35 mm glass bottomed dishes. Continue to use the 63x 1.2NA water immersion objective.
2. Apply a small drop of water immersion medium to the objective, and place the dish with mounted larva on the stage. Stabilize the dish with clips.
3. Using the eyepiece and widefield illumination, focus the larva and locate the motor nerves. Scan the nerves in hemisegments 10-20 and select a motor nerve for transection.
4. Bring up a live-view of the nerve on the computer screen using imaging software. Select Z-planes and acquire an image of the axons and glial cells of the uninjured nerve.
5. Prepare time-lapse imaging settings in the imaging software to capture Z-projections of all cell types in 5-30 min intervals, depending on the experiment. It is best to create all necessary settings for the time-lapse before performing the ablation, so there is no delay in starting the time-lapse once the injury is complete.
6. Remove the "100%ILL" beam splitter and replace with the "blank". Close the light path to the oculars.
7. Return to the live-view of the nerve. Use the appropriate fluorescent channel to view the axons that will be transected.
8. Using the ellipse tool, create a thin elliptical ROI in the area to be ablated, then create smaller ROIs within the selected region.
9. In the window that controls the laser settings, set the number of pulses to "2" and the attenuation plate to "18" % transmission. Fire the laser within the selected ROIs. If fluorescence remains within the ROIs, increase the laser power, wait approximately 10 sec, and fire the laser again. Do this until you reach a setting that causes fluorescence to disappear within the ROIs.
10. Wait 10 or more seconds and check the ablated area again for fluorescence. Beware that an ROI may initially appear ablated, when it is actually photobleached. If fluorescence returns, increase the laser power and fire the laser again. Repeat this until florescence disappears within the ROIs and does not return within 10 sec. It is best to start with a lesser laser power and increase to the necessary power. The necessary laser power may vary based on individual microscopy systems, age of Coumarin dye, specimen mounting, age of larvae, and thickness of the tissue. Once an ideal laser power has been established, this power setting may be used again on subsequent nerves in the same experiment.
11. Once the ablation is completed, begin time-lapse imaging.
12. When the time-lapse is complete, use imaging software to compile data and create color composite Z-projections for each time point. Create a QuickTime movie to analyze the behavior of axons and glial cells simultaneously .

Representative Results

The assay described here can be used to assess the response of glial cells and other nerve-associated cell populations to axonal injury *in vivo*. **Movie 1** shows an example of a nerve injury created using this method and the response of surrounding glial cells. This experiment was performed in *Tg(nkx2.2a:megfp);Tg(olig2:dsred)* zebrafish, in which perineurial glia express a membrane targeted EGFP and motor neurons express cytosolic DSRed. The injury was made along the rostral projection of a trunk motor nerve in a 6 dpf live zebrafish, and the nerve was subsequently time-lapse imaged in both the EGFP and DSRed channels. This allowed simultaneous visualization of axon and glial cell behaviors immediately following the injury.

Figure 1 shows still images of static time-points taken from **Movie 1**. The dotted ellipse shows the ROI that was ablated using the laser. One minute post transection (mpt), the ablated area lacked fluorescence and the injury zone measured approximately 3.5 um from the proximal to distal stump. The success of a transection can be confirmed by imaging the distal nerve stump and looking for signs of Wallerian degeneration, including distal axon fragmentation and rapid clearance. The absence of axonal fluorescence along the distal stump in **Figure 1** at 120 mpt indicates these axons have indeed undergone Wallerian degeneration and the transection was successful.

Adjusting the laser power to an ideal setting is critical when performing laser ablation experiments. Ideal laser power settings will cleanly ablate the nerve only within the selected ROI, and laser power settings that are either too low or too high will yield suboptimal results. **Figure 2a** shows an injury that was performed with a laser power that was too low. Fluorescence remained within the ROI after firing the laser, resulting in an incomplete transection. **Figure 2b** shows an injury that was performed with a laser power that was too high, resulting in an extremely large ablation.

Movie 1. Motor nerve transection and confocal time-lapse imaging of perineurial glial cell behavior. Movie shows a trunk motor nerve pre-injury, followed by an image taken 1 mpt, and images every 10 min for a total of 190 min. Injury was performed in a live 6 dpf *Tg(nkx2.2a:megfp);Tg(olig2:dsred)* zebrafish larva mounted with anterior to the left and dorsal to the top. Click here to view movie.

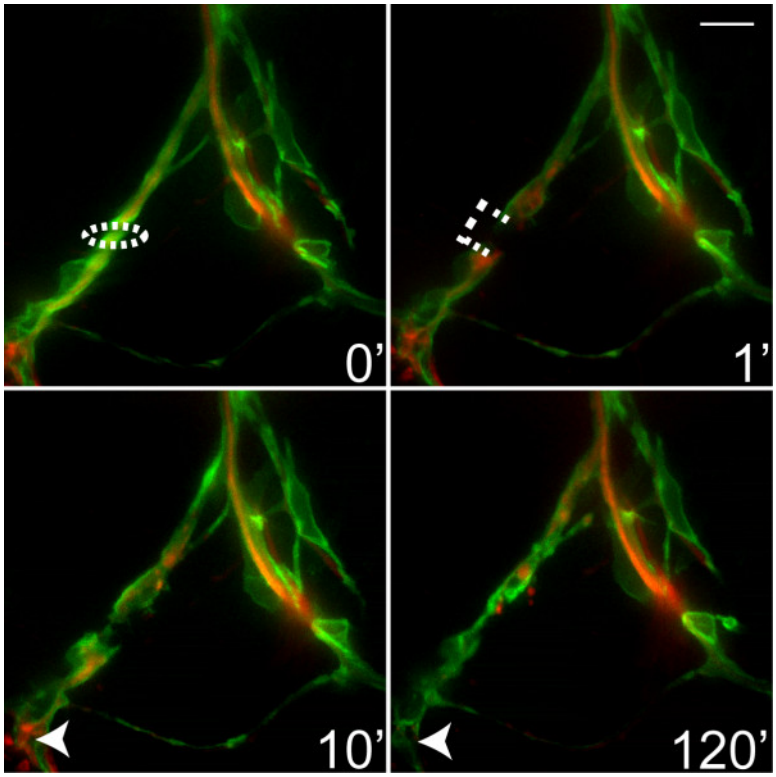

Figure 1. Motor nerve transection and still images of glial cell behavior. Panels are still images taken from **Movie 1**. The dotted elliptical area represents the ROI that was ablated, creating a transection injury denoted by the dotted line. Arrowheads point to axonal fluorescence that is present at 10 mpt, but not at 120 mpt, indicating the distal axons degenerated. Scale bar, 10 μm.

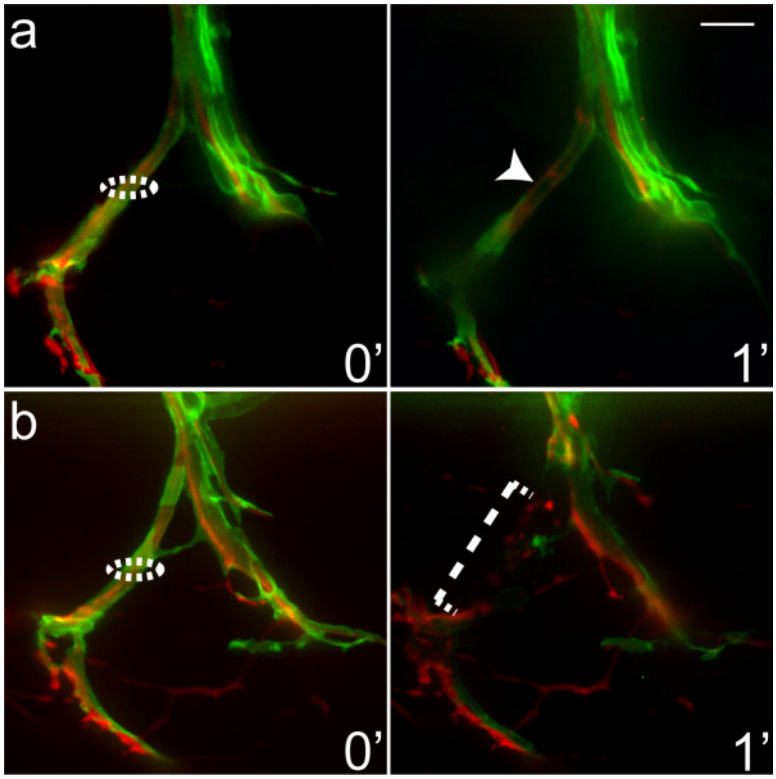

Figure 2. Suboptimal laser power settings leads to undesired results. (a) An attempted ablation performed with a laser power that is too low. The dotted ellipse denotes the selected ROI. 1 mpt the area has been slightly photobleached and not transected (arrowhead). **(b)** An ablation performed with a laser power that is too high. The dotted ellipse denoted the selected ROI. 1 mpt the ablated area is much larger than the selected ROI (dotted line). All images are taken from live 6 dpf *Tg(nkx2.2a:megfp);Tg(olig2:dsred)* zebrafish larvae with anterior to the left and dorsal to the top. Scale bar, 10 µm.

Discussion

The most critical steps of this experimental design are: 1) properly mounting larvae for injury and subsequent *in vivo* imaging and 2) calibrating the laser and selecting the correct power settings in order to create a clean nerve transection that results in minimal extra-tissue damage. To help ensure a successful axotomy for *in vivo* imaging and subsequent analysis, mount multiple larvae in either individual glass bottom dishes or in a glass bottom dish with dividers. After calibrating the laser, we recommend testing different power settings on a test larva to identify the optimal parameters for nerve transection. These settings are usually fairly consistent between experiments, but can change if: 1) larvae are not mounted properly, 2) larvae of different ages are used, 3) the laser isn't calibrated correctly, 4) the laser needs maintenance or 5) if nerves are located in very different anterior-posterior positions which would result in a difference in tissue thickness. An optimal nerve transection will create an injury along a nerve that is between 2 and 5 µm and doesn't disturb neighboring tissue.

When analyzing the response of any cell type to nervous system injury, we suggest conducting at least 2 types of controls. The first is selecting an area to injure that is immediately next to the nerve of interest, but is not touching any nervous tissue. This will allow you to determine if the cellular responses you see are due to damage in general, or to injury of axons. The second control is to image an uninjured nerve in the same fish that you have created an injury. This type of control eliminates larva to larva variability due to staging and imaging parameters, including confocal exposure times for imaging, etc.

In this protocol we also describe scenarios that create injuries that are too small or too large for our particular studies. Depending on the experimental question, these types of injuries can be used for analysis. The main limitations to this type of procedure would be the objectives available for creating the injury and subsequent time-lapse imaging. The older the larva you use, the longer working distance objective required.

The protocol we describe here allows the user to create focal nerve transections along deep nerves in mature larvae. We couple this technology to *in vivo*, time-lapse imaging and use a commercially available laser ablation system that reproducibly creates focal axon injuries. This technology can be altered to use different transgenic lines or mutant larvae to test different hypotheses about the mechanisms that rebuild the nervous system after injury.

Disclosures

The authors have nothing to disclose.

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