BSU Honors Program

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Technique for Staining the Myelin-like Sheath in Earthworms

Biology

07 May 2014

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Honors Thesis 2014

Bemidji State University

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Abstract:

Not only are worms known for digesting dirt, but they are also famous for their unique regenerative properties. Previously published work has demonstrated that with certain enhancement factors, earthworms can regenerate 90% of nerve cells after injury. A form of myelin, a specialized sheath comprised of cellular membranes that have an uncharacteristically high lipid to protein ratio, surrounds the main nerve cord in earthworms. While it is still unknown what regenerative properties the sheath has, this project describes the creation and execution of a procedure involving cross section preparation, staining, and visual examination for the myelin-like sheath in earthworms.

Background:

Within the human brain, spinal cord, and peripheral nerves are two cell types: neurons and glia. The cellular projections of many neurons require insulation to properly conduct impulses. This essential insulation comes from a fatty substance called myelin, a specialized sheath comprised of glial cell membranes that have an uncharacteristically high lipid to protein ratio. While an average cell membrane, such as that of a skin cell or a neuron, is composed of approximately 20% lipids and 80% protein. The cell membranes of glial cells are 79% lipids and 18% protein (Guidotti, 1972). Accumulation of these lipids is what makes the white matter white. Two types of glial cells, oligodendrocytes and Schwann cells are the only two cell types in vertebrates capable of manufacturing myelin. Oligodendrocytes

are located in the central nervous system (CNS), which consists of the brain and spinal cord. Schwann cells are found in the peripheral nervous system (PNS), which consists of the nerves protruding from the spinal cord and extending throughout the body. Together, the CNS and PNS control our body's every thought, sensation, and movement.

Proper functioning of the nervous system requires myelin for increasing the speed of nerve signal transmission. When signals, within the brain and those sent out to other parts of the body, are disrupted, the body cannot function as it normally would. Thus, compromised myelin commonly leads to symptoms including: fatigue, weakness of extremities, impairment of vision and speech, memory loss, and incontinence. These symptoms are found in autoimmune and other diseases that result from demyelination, or loss of the myelin sheath, and the lesions that are left behind. Multiple sclerosis (MS), Adrenoleukodystrophy (ALD), and Gullian-Barré Syndrome are a few examples of neurodegenerative autoimmune diseases. The prevalence of these tragic, sometimes fatal diseases provides an obvious clinical application and need for myelin repair research.

Remyelination studies in rats and other small mammals have had encouraging results. In a study conducted on Schwann cell remyelination, rat spinal cords were injured and transplanted with Schwann cells that were found to enhance remyelination, though the resulting regenerated myelin sheath was thinner than that of the uninjured control rat spinal cords (Zhang et al, 2013). Another study on rodents demonstrated remyelination

was enhanced by exposure to hormones and vitamins. Thyroid hormone, essential for neural development, played a key role in the synthesis of new myelin. Thyroid hormone regulates the expression of cytoskeleton proteins during axon regeneration, which is essential for white matter (myelin) development (Calza et al., 2010). Additionally, there was another study on mice using quetiapine, an antipsychotic drug, to enhance oligodendrocyte development and remyelination. This study suggests quetiapine upregulates oligodendrocyte populations in the demyelinated lesions and may play a role in improving cognitive function (Zhang et al., 2012). While there have been some successes in small mammals, there is difficulty in transitioning this knowledge back to the complex nervous system of humans.

Going forward, there are multiple reasons that make the earthworm an excellent candidate for myelin research. Earthworms are easily obtained, cost effective, and simple to house in the lab. They also contain a single giant axon with regenerative properties and have a sequenced and partially annotated genome. With its documented ability to regenerate its giant axon, the earthworm may be an ideal model to study repair of the myelin-like sheath at a more basic level.

Research Question:

In the Bemidji State University biology laboratory under the instruction and guidance of Dr. Angela Hahn, my research stemmed from the question: Is it possible to visually detect the myelin-like sheath of the earthworm giant

axon using a simple staining procedure and BSU's transmitted light microscopes?

Methods:

Dissection:

Earthworms (*Lumbricus rubellus)* were obtained from a local bait shop and cut into sections roughly one inch in length. The following steps used either the full tissue sections or the dissected giant axons. It should be noted that earthworms do not have pain receptors (Somme 2005), and all specimens were treated respectfully during this project.

Fixation:

Fixation is a necessary step to prevent degradation of the tissues. The fixative consists of 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS), pH 7.4 and the ideal time for fixing the tissues is approximately 48 hours at 4°C.

Embedding in Paraffin Wax:

After fixation in 4% paraformaldehyde, the worm sections are washed six times with PBS and then embedded in paraffin wax. Paraffin wax beads were melted in an electrical warmer and then pipetted, using disposable pipets, into the plastic cassettes containing the fixed worm sections. Paraffin wax is similar to candle wax and acts as a stabilizer for the tissues when being cut with the microtome.

Cross Sectioning:

The wax blocks containing either worm segments or giant axons were then cross sectioned using the American Optical Cryo-Cut Cryostat Microtome (Fig. 1) at approximately 15 microns in thickness. Worm sections enclosed in the wax were placed in the object clamp. The desired width was adjusted on the thickness indicator. With the blade properly inserted in the knife holder, the crank was turned clockwise and cross sections were shaved off of the wax block. Cross sections were stored in wells containing the 4% paraformaldehyde fixative solution.

Figure 1: American Optical Cryo-Cut Cryostat Microtome

Staining:

The cross sections were stained following the Luxol Fast Blue - Cresyl Echt Violet Stain Kit Procedure (Item# KTLFB, American MasterTech scientific lab supplies). The luxol fast blue stain was heated in a 60°C water bath, then the cross sections were added to the preheated stain and incubated for 1 hour. Next, the cross sections were rinsed with distilled water and dipped several times in 0.05% lithium carbonate followed by two changes of 70% reagent alcohol. After these steps, myelin should be blue to

turquoise. The cross sections were rinsed again with distilled water and counterstained for nissl substances with the cresyl echt violet stain for 10 minutes. Another distilled water rinse followed. The cross sections were then dipped five to ten times in 70% reagent alcohol to continue differentiating the tissues. To dehydrate the cross sections, they went quickly through 3 changes of absolute alcohol, as excess time will decolorize cresyl echt violet stain. The stained cross sections were stored in wells in PBS at 4ºC.

Microscope Analysis & Imaging:

The stained cross sections were examined for the presence of myelin using regular light microscopes at various magnifications. Images of the cross sections were taken through the microscope using cellular phones (Fig. 3).

Results:

With the staining procedure used in this experiment, myelin is stained blue/turquoise whereas the endoplasmic reticulum and nuclei stain purple. Based on the diagram of the earthworm cross section (Fig. 2) I identified the structures of the stained tissues (Fig. 3). The ventrally-located nerve cord or giant axon in the diagram is encircled by a dashed line in the stained cross section.

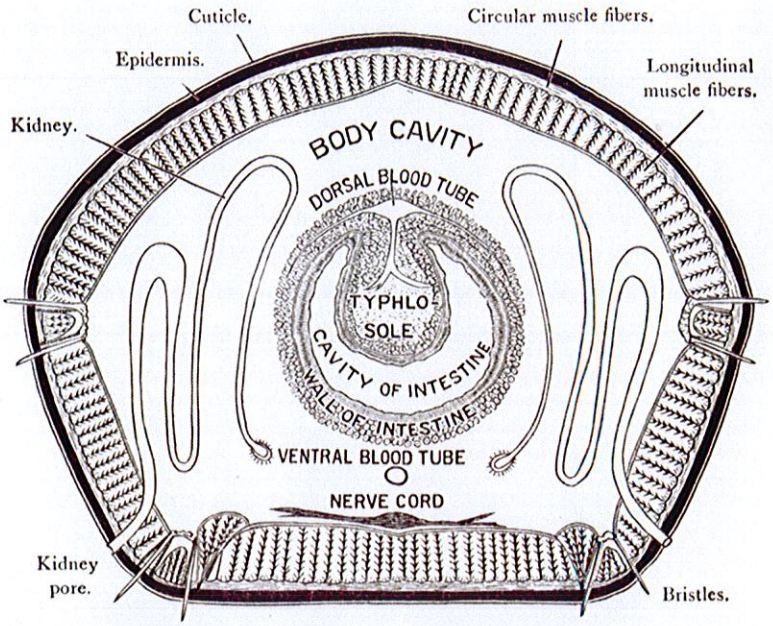

Figure 2: Anatomy of an earthworm cross section

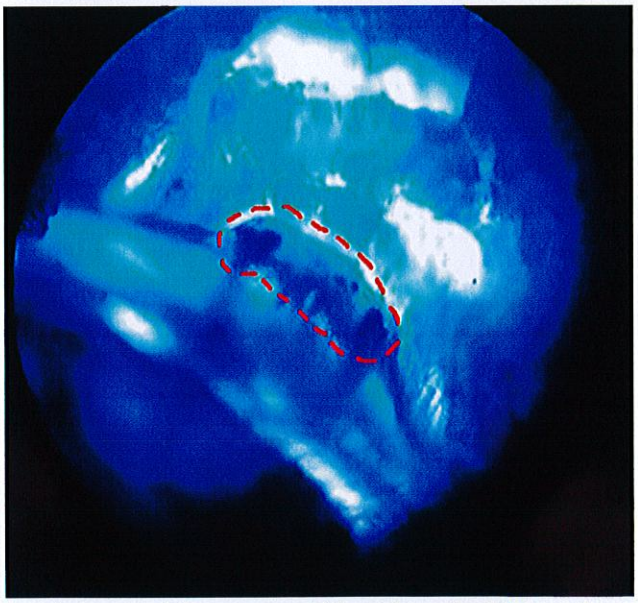

Figure 3: Cross section at 40x magnification (within the dashed line is the ventral nerve cord)

Discussion & Conclusion:

As previously mentioned, the giant axon is encircled by the dashed line in Fig. 3. Areas of darker blue stain can be visually depicted within and

extending from the nerve cord, which I believe to be a myelin-like substance.

This preliminary research project sets the stage for future studies. Perhaps the first could be examining the myelin-like sheath of earthworms after injury; this could be done by severing the giant axon, allowing time for regeneration, and following the same procedure to obtain stained cross sections. Additionally, leeches could be tested alongside the earthworms to act as a negative control; leeches are evolutionarily similar to earthworms but do not contain any myelin-like tissues.

Furthermore, I would like this work to encourage the updating of lab equipment. A newer microtome would provide more consistent tissue slices, and disposable blades with a blade holder would allow for thinner cross sections consistently and over an extended period of time. In order for the stains to completely penetrate the cross section tissues, 8 microns is the ideal thickness; BSU's microtome could produce cross sections of a minimum thickness of 15 microns and no thinner. Microtome blades are unique and cannot be sharpened by anyone other than the manufacturer. As a result, a temporary disposable blade was fastened to the original blade. Doing this allowed for sharper cuts without having to purchase a new specialized blade, but it was obviously not ideal.

Another hurdle was keeping the paraffin wax block at an optimal temperature. It must stay cold enough to remain rigid, but if it becomes too cold, it will crack. Placing the wax in the freezer caused it to crack during

cutting with the microtome. A recommendation would be to keep the wax blocks in an ice bath when not being cut.

In conclusion, I was able to stain the myelin-like sheath in earthworm cross sections obtained with a 1960's microtome. Thus, the opportunity does exist for future earthworm myelin regeneration studies to be executed at Bemidji State University.

Acknowledgements:

I would like to thank my advisor, Dr. Angela Hahn, for her inspiration in designing this project, helpful knowledge of lab techniques, and encouragement to continue studying the wonders of science.

Also, thank you to Dr. Michael Hamann for advice on using "The Beast" and getting it removed from its original hefty home.

Lastly, special thank yous to the 2013-2014 Advanced Lab Projects students and graduate students for making unforgettable memories in lab.

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Figure 1: Microtome - Kayla Brown & Chantelle Koppe

Figure 2: Earthworm Cross Section Anatomy http://etc.usf.edu/clipart/6900/6940/earthworm_6940_md.gif

Figure 3: Stained Cross Section - Kayla Brown & Chantelle Koppe

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