NERVE FIBER DIAMETER MEASUREMENTS USING HEMATOXYLIN AND EOSIN
STAINING AND BRIGHTFIELD MICROSCOPY TO ASSESS THE NOVEL
METHOD OF CHARACTERIZING PERIPHERAL NERVE
FIBER DISTRIBUTIONS BY GROUP DELAY

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TITLE: Nerve Fiber Diameter Measurements Using Hematoxylin and Eosin Staining and Brightfield Microscopy to Assess the Novel Method of Characterizing Peripheral Nerve Fiber Distributions by Group Delay

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ABSTRACT

Nerve Fiber Diameter Measurements Using Hematoxylin and Eosin Staining and Brightfield

Microscopy to Assess the Novel Method of Characterizing Peripheral Nerve

Fiber Distributions by Group Delay

Jorge Arturo Vazquez

Peripheral neuropathies are a set of common diseases that affect the peripheral nervous system, causing damage to vital connections between various parts of the body and the brain and spinal cord. Different clinical conditions are known to selectively impact various size nerve fibers, which often makes it difficult to diagnose which peripheral neuropathy a patient might have. The nerve conduction velocity diagnostic test provides clinically useful information in the diagnosis of some peripheral neuropathies. This method is advantageous because it tends to be minimally invasive yet it provides valuable diagnostic information. However, this test does not determine characteristics of peripheral nerve fiber size distributions, and therefore does not show any detailed information regarding the nerve fibers within the nerve trunk. Being able to determine which nerve fibers are contributing to the evoked potential within a nerve trunk could provide additional information to clinicians for the diagnosis of specific pathologies of the peripheral nervous system, such as chronic inflammatory demyelinating polyneuropathy or early diabetic peripheral neuropathy. In this study, three rat sciatic nerves are sectioned and stained with hematoxylin and eosin in order to measure the nerve fiber diameters within the nerve trunk. Stained samples are viewed using brightfield microscopy and images are analyzed using ImageJ. Histograms were created to show the frequency of various nerve fiber diameters. The nerve fiber diameters measured during this research are consistent with the range of previously published diameter values and will be used to support continuing research for a novel method to characterize peripheral nerve fiber size distributions using group delay.

Keywords: Group delay, peripheral neuropathy, neuron, hematoxylin, eosin, histology,

nerve fiber size, fiber diameter, distribution conduction velocity, nerve conduction velocity

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CHAPTER I: INTRODUCTION

An estimated 15 to 20 million people in the United States over age 40 have some type of peripheral neuropathy [1]. However, many patients are unaware that they have a disease, are not given the correct diagnosis, or the diagnosis is delayed. Currently, treatments do not fix the actual nerve fiber dysfunction or fiber loss, but are focused on treating the underlying medical conditions that cause the neuropathy or are focused on treating symptoms [2]. Some helpful diagnostic techniques include skin biopsies, quantitative sudomotor axon reflex testing (QSART), nerve conduction studies, and electromyography. The skin biopsy and QSART are objective, reproducible, sensitive, and complementary however, these two tests are not widely available. The electrodiagnostic techniques are advantageous because they tend to be minimally invasive yet provide valuable diagnostic information [1].

The nerve conduction velocity test provides clinically useful information in the diagnosis of peripheral neuropathies, such as carpal tunnel syndrome [3]. During the nerve conduction velocity test, surface electrode patches are placed on the surface of the skin to stimulate a specific nerve. One electrode stimulates the nerve while the other records when the compound evoked potential reaches its position. This test determines the speed of conduction of a compound evoked potential down the nerve. While the peripheral nerve conduction velocity test can provide useful information to the clinician regarding the viability of the nerve under study, it is a single-parameter test that yields no detailed information about the characteristics of the functioning nerve fibers within the nerve trunk [3]. Being able to determine information regarding the functioning nerve fibers within the nerve trunk could potentially help differentiate between different clinical conditions such as chronic inflammatory demyelinating polyneuropathy, which selectively impacts larger nerve fibers, or early diabetic peripheral neuropathy, which impacts smaller fibers [4,5].

Szlavik et al [3] presented a novel technique for estimating the size distribution of contributing nerve fibers which is linearly related to the conduction velocity distribution. The technique is based on an estimation of the group delay between two sets of recording electrodes associated with the individual fibers that contribute to a maximal compound-evoked potential. Incrementally stepping up the stimulation current using small enough steps will, in theory, recruit more and more nerve fibers until a maximal compound evoked potential is reached. With each additional fiber recruited, the output signal increases in magnitude. As the stimulation current continues, the increment is so small that only one new fiber gets recruited with each new step. Then, subtracting the previous compound evoked potential from the current one will yield the potential associated with a single fiber. The group delay information is then used to estimate the diameters of the activated fibers as well as the propagation delays of individual single-fiber-evoked potentials to a reference electrode. This process allows for reconstruction of an estimated maximal compound evoked potential, from the individual single-fiber-evoked potentials, at the first recording site. The results of the simulation study demonstrated that the technique could retrieve the fiber size distribution in the presence of recording noise for a wide range of signal-to-noise ratio (SNR) values.

The aforementioned technique has inspired future research regarding the efficacy of the novel method for characterization of peripheral nerve fiber size distributions by group delay. This thesis project will use three extracted rat sciatic nerve, one control model and two chemically-induced diabetes models, for the purpose of sectioning and staining the nerves in order to determine the individual nerve fiber diameters. These values will then be compared to results from the study of characterizing peripheral nerve fiber size distributions by group delay, to determine the efficacy of using this test to characterize different peripheral neuropathies.

CHAPTER II: BACKGROUND

ANATOMY AND PHYSIOLOGY OF A NERVE

THE NERVOUS SYSTEM

The nervous system is one of the most complex systems of the 11 body systems, made up of a highly organized network of billions of highly specialized nerve cells (neurons) and cells that support the activities of neurons (neuroglia). The structures that make up the nervous system include the brain, spinal cord, cranial nerves and their branches, spinal nerves and their branches, ganglia enteric plexuses, and sensory receptors. These structures can be separated into two main subdivisions: the Central Nervous System (CNS), which consists of the brain and spinal cord, and the Peripheral Nervous System (PNS), which includes all nervous tissue outside the CNS and connects the CNS to other body parts [Figure 1].

Figure 1. The CNS includes the brain and spinal cord, while the PNS includes all nervous tissue outside the CNS and connects the CNS to other body parts [7]

The nervous system carries out a complex array of tasks which can be grouped into three basic functions: sensory, integrative, and motor.

- *Sensory function:* Sensory receptors at the ends of peripheral neurons detect internal stimuli, such as an increase in blood acidity, and external stimuli, such as a raindrop landing on your arm. Sensory receptors convert their information into nerve impulses, which are then carried to the brain and spinal cord through the cranial and spinal nerves of the PNS.
- *Integrative function:* Once the signals reach the CNS, they are integrated (processed), creating sensations, adding to memory, or helping produce thoughts. An important integrative function is perception, the conscious awareness of sensory stimuli.
- *Motor function:* Following integration, conscious or subconscious decisions are made and then acted upon by means of motor functions. The nervous system may elicit an appropriate motor response by activating effectors (muscles and glands) through cranial and spinal nerves. Stimulation of the effectors causes muscles to contract and glands to secrete. The motor portion of the PNS can be subdivided into the somatic nervous system (SNS), autonomic nervous system (ANS), and enteric nervous system (ENS) [6, 7].

The SNS consists of sensory neurons that convey information from somatic receptors in the head, body wall, and from receptors for the special senses of vision, hearing, taste, and smell to the CNS; and motor neurons that conduct impulses from the CNS to skeletal muscles only. The action of the SNS is considered voluntary since the motor responses can be consciously controlled. The ANS consists of sensory neurons that convey information from autonomic sensory receptors, primarily in visceral organs, to the CNS; and motor neurons that conduct nerve impulses from the CNS to smooth muscle, cardiac muscle, and glands. The action of the ANS is considered involuntary because motor responses are not normally under conscious control. The

ENS consists of neurons that extend most of the length of the gastrointestinal (GI) tract. Sensory neurons of the ENS monitor chemical changes within the GI tract as well as the stretching of its walls. The ENS is considered the “brain of the gut”, and its action is considered involuntary [7].

HISTOLOGY OF NERVOUS TISSUE

Nerve tissue consists of two types of cells: neurons and neuroglia. Neurons sense certain types of changes in their surroundings and respond by transmitting nerve impulses along cellular processes to other neurons or to muscles or glands, resulting in coordination, regulation, and integration with many body functions. Neurons provide most of the unique functions of the nervous system, such as sensing, thinking, remembering, controlling muscle activity, and regulating glandular secretions. The abundant neuroglia support and bind the components of nervous tissue, carry on phagocytosis, and help supply nutrients to neurons by connecting them to blood vessels [6].

Neurons vary considerably in size and shape, but they all have three basic parts: a cell body, dendrites, and an axon [Figure 2]. The cell body, or soma, contains a large spherical nucleus surrounded by cytoplasm that includes typical cellular organelles such as mitochondria, lysosomes, a Golgi apparatus, and many microtubules. Extending from the soma are processes called nerve fibers. Most neurons have two kinds of nerve fibers, dendrites and an axon. Dendrites are usually highly branched processes extending from the cell body and are the receiving or input portions of a neuron. The cell body can have many dendrites, but only one axon extends from the soma. The axon is a long, thin, cylindrical projection that arises from the cell body at a cone-shaped elevation called the axon hillock. The axon is responsible for propagating nerve impulses away from the cell body toward another neuron, a muscle fiber, or a gland cell.

Figure 2. Structure of a multipolar neuron - a neuron with a large cell body, several short dendrites, and a single long axon. Arrows indicate the direction of information flow: dendrites --> cell body --> axon --> axon terminals [7]

Neuroglia of the PNS completely surround axons and cell bodies and are grouped into two cell types: Schwann cells and satellite cells. Schwann cells encase the larger axons of peripheral neurons in lipid-rich sheaths formed by tightly wound layers of cell membrane [Figure 3]. The layers are composed of a myelin sheath, which has a higher proportion of lipid than other surface membranes. Axons that have myelin sheaths are called myelinated axons, and those that lack these sheaths are called unmyelinated axons. The myelin sheath electrically insulates the axon of a neuron and increases the speed of nerve impulse conduction.

Figure 3. Schwann cells of the PNS completely surround axons and cell bodies of neurons [7]

Schwann cells are responsible for the production of myelin sheaths in the PNS, beginning during fetal development. Each Schwann cell wraps about 1 millimeter of a single axon's length by spiraling many times around the axon [Figure 4]. Up to 100 layers of Schwann cell membrane becomes the myelin sheath, with the Schwann cell's cytoplasm and nucleus forming the outermost layer, the neurolemma. The neurolemma aids regeneration when an axon is injured by forming a regeneration tube that guides and stimulates regrowth of the axon. Narrow gaps in the myelin sheath between Schwann cells are called nodes of Ranvier [Figure 2]. The nodes of Ranvier play a key role during the propagation of action potentials.

Figure 4. Transverse sections of stages in the formation of a myelin sheath [7]

NERVE COMMUNICATION

Neurons communicate with one another using two types of electrical signals: Graded potentials for short-distance communication and action potentials for long-distance communication. The production of graded potentials and action potentials depends on the presence of specific types of ion channels on the plasma membrane that open and close in response to specific stimuli, and on the existence of a membrane potential which is an electrical potential difference (voltage) across the membrane.

Potassium ions (K^+) are the major intracellular positive ion, while sodium ions (Na^+) are the major extracellular positive ion. The distribution is created largely by the sodium-potassium pump, which actively transports sodium ions out of the cell and potassium ions into the cell. The distribution of ions is also affected by channels in the cell membrane that determine membrane permeability. In the case of a resting neuron, the membrane potential is called the resting membrane potential and has a typical value of -70 mV. The negative sign indicates the excess negative charges on the inside of the cell membrane [Figure 5]. Sodium and Potassium ions continue to diffuse across the cell membrane at a rate of three sodium ions leaking into the cell for every two potassium ions that leak out, which is then balanced by the sodium-potassium pump.

Figure 5. Distribution of charges that produce the resting membrane potential of a neuron [7]

An action potential is a sequence of rapidly occurring events that decrease and reverse the membrane potential and then eventually restore it to the resting state. The phases that occur during an action potential include the: depolarization phase, repolarization phase, after-hyperpolarization phase, and refractory period. In order for depolarization to occur, a threshold stimulus must occur to bring the membrane potential to a threshold value (-55 mV in many neurons). Once the threshold value is reached, the depolarization phase begins where voltage-gated sodium channels open rapidly and cause an inrush of sodium ions. The inflow of sodium ions changes the membrane potential to +30 mV, meaning the inside of the membrane is 30 mV more positive than the outside. These voltage-gated sodium channels close quickly, but almost simultaneously, slower voltage-gated potassium channels open and briefly increase potassium permeability. The slowing of sodium inflow and the acceleration of potassium outflow causes the repolarization phase, which causes the inside membrane potential to change from +30 mV to -70 mV. While the voltage-gated potassium channels are open, outflow of potassium may be large enough to cause an after-hyperpolarization phase of the action potential, where the membrane potential may become as negative as -90 mV. As the voltage-gated potassium channels close, the membrane potential returns to the resting level of -70 mV and remains in the resting state until it is stimulated again.

Figure 6. Main phases of an action potential [7]

Three major factors that affect the speed of propagation of an action potential include: amount of myelination, axon diameter, and temperature. Action potentials travel, or propagate, more rapidly along myelinated axons than along unmyelinated axons, due to saltatory conduction. Saltatory conduction is when action potentials only occur at the nodes of Ranvier, and therefore appear to “leap” between long segments of myelinated axolemma. Jumping from node to node makes current flow much faster than in an unmyelinated axon of the same diameter. Saltatory conduction also represents a more energy-efficient mode of conduction since less ATP is used by sodium-potassium pumps to maintain the intracellular and extracellular balance of ions. The diameter of the axon also affects the speed of nerve impulse conduction – larger diameter axons propagate action potentials faster than smaller ones. This is because larger diameter axons have a greater total volume for charges to flow through and correspondingly meet less internal resistance from the membrane. Temperature also affects the speed of propagation – higher temperatures result in higher propagation speeds [7].

CLASSIFICATION OF NERVE FIBERS

Axons can be classified into three major groups based on the amount of myelination, the diameter, and the propagation speeds. *Table 1* shows the various types of fibers and some of their characteristics.

Table 1. Nerve Fiber Types and Characteristics [7]

Fiber Type	Diameter Size (μm)	Propagation Speed	Function
A Fibers (Myelinated) <ul style="list-style-type: none"> • $A\alpha$ • $A\beta$ • $A\gamma$ • $A\delta$ 	5 – 20	12 – 130 m/sec (27 – 280 mi/hr)	Sensory neurons that propagate impulses associated with touch, pressure, position of joints, and some thermal and pain sensation
B Fibers (Myelinated)	2 – 3	< 15 m/sec (< 32 mi/hr)	Conduct sensory nerve impulses from the viscera to the brain and spinal cord
C Fibers (Unmyelinated)	0.5 – 1.5	0.5 – 2 m/sec (1 – 4 mi/hr)	Conduct some sensory impulses for pain, touch, pressure, heat and cold from the skin, and pain impulses.

These nerve fiber types can then be further classified according to size into large, medium, or small fibers, which also correlates with the degree of myelination. Large nerve fibers are heavily myelinated and include A-alpha fibers and A-beta fibers. Medium-sized fibers are known as A-gamma fibers. Small fibers include myelinated A-delta fibers and unmyelinated C-fibers [1].

NERVE DAMAGE

DAMAGE AND REPAIR

Injury to the cell body usually kills a mature neuron, however, a damaged peripheral axon may regenerate depending on the damage. Axons and dendrites that are associated with a neurolemma may undergo repair if the cell body is intact, if the Schwann cells are functional, and

if scar tissue formation does not occur too rapidly [6, 7]. When there is injury to an axon or a disease process, changes will occur in the cell body and in the portion of the axon distal to the site of injury. By the third to fifth day, the part of the axon distal to the damaged region and the myelin sheath deteriorate. This debris is phagocytosed by macrophages. A regeneration tube may be formed across the injured area by two Schwann cells joining on either side of the injured site. This tube guides growth of a new axon across the injured area toward the distally located receptors and effectors. Thus, some sensory and motor connections are reestablished and some functions restored. Eventually, remaining Schwann cells proliferate along the length of the degenerating portion and form new myelin around the growing axon.

PERIPHERAL NEUROPATHY

Peripheral neuropathy describes damage to the peripheral nervous system, which damages vital connections between various parts of the body and the brain and spinal cord. Peripheral neuropathy may be caused by physical trauma to nerve, autoimmune responses, systemic disease, or genetic errors. Different clinical conditions are known to selectively impact various size fibers. Peripheral neuropathy can be categorized based on the function of the involved nerve fibers or on their diameter and conduction velocity.

Small fiber dysfunction can be defined as a generalized peripheral neuropathy in which the small diameter myelinated and unmyelinated nerve fibers are affected, either exclusively or to a much greater degree than the large diameter myelinated fibers [8]. Small fiber neuropathy (SFN) has been associated with many medical conditions including glucose dysmetabolism, connective tissue disease, HIV infection, hereditary diseases, alcoholism, and others [1]. Additional underlying diseases are shown in *Table 2*. Nerve conduction studies and electromyography, which are primarily used for the evaluation of large fiber neuropathies, are generally normal diagnostic procedures for patients with SFN as well. However, it is difficult to diagnose SFN since, in many patients, the results of nerve conduction studies and EMG are

normal. The normal results cause SFN to be easily overlooked or misdiagnosed [1]. These tests are often performed anyway in order to rule out subclinical involvement of large fibers. Although there are diagnostic techniques available, they are not widely available; therefore it has been difficult to establish a gold standard for the evaluation of SFN.

Table 2. (Idiopathic) Causes of small fiber neuropathy [9]

Inherited	Familial Amyloidosis
	Autosomal recessive hereditary neuropathy
	Fabry's disease
	Hereditary sensory and autonomic neuropathy
	Ross syndrome
	Friedreich's ataxia
	Tangier disease
Acquired	Diabetes mellitus
	Impaired glucose tolerance
	Alcoholism
	Systemic amyloidosis
	Vasculitis
	Sarcoidosis
	Sjögren's disease
	Systemic lupus erythematosus
	Guillain-Barre syndrome
	Antecedent viral infection
	HIV
	Antisulfatide antibodies
	Hyperlipidemia
	Complex regional pain syndrome
	Paraneoplastic syndrome
	Neurotoxic medication

Chronic inflammatory demyelinating polyneuropathy (CIDP) is a neurological disorder characterized by progressive weakness and impaired sensory function in the legs and arms. It is an example of a large fiber peripheral neuropathy since it is caused by damage to the myelin sheath of the large peripheral nerves. CIDP is closely related to Guillain-Barre syndrome and it is considered the chronic counterpart of that acute disease. Electrodiagnostic testing is recommended for all patients with suspected CIDP. Additional diagnostic evaluations include cerebrospinal fluid analysis, nerve biopsy, Magnetic Resonance Imaging (MRI) of spinal roots, laboratory studies, and evaluation for inherited neuropathies. While these diagnostic evaluations can be helpful to the clinician, none have ideal sensitivity and specificity.

DIAGNOSTIC TECHNIQUES

LABORATORY EXAMINATIONS

Most experts recommend cerebrospinal fluid analysis in order to demonstrate the typical findings in several peripheral neuropathies: increased protein and a normal or only slightly elevated cell count [10]. More extended laboratory testing may also be necessary in some patients to search for causes of demyelinating polyneuropathy, as well as concurrent diseases.

NERVE BIOPSY

The diagnostic value of nerve biopsy has been extensively debated, with some experts believing that there is no diagnostic value, whereas others view it as essential for diagnosis and management in up to 60 percent of patients with chronic demyelinating polyneuropathy [10]. Nerve biopsies have a low diagnostic yield in chronic inflammatory demyelinating polyneuropathy, for several reasons. The most prominent abnormalities may lie in the proximal segments of the nerves or roots or in motor nerves, which are areas not accessible to biopsy. Also, secondary axonal changes starting early in the disease processes may over-shadow the initial signs of demyelination and inflammation by the time a biopsy is performed.

MRI

MRI may be used to demonstrate gadolinium enhancement and enlargement of proximal nerves or roots, reflecting active inflammation and demyelination in the caudal equine or brachial plexus [10]. Abnormalities of the brachial plexus with irregular swelling and increased signal intensity were detected in about 50 percent of patients with CIDP.

CONTACT HEAT-EVOKED POTENTIALS

Contact heat-evoked potentials (CHEP) is a non-invasive assessment to study the function of painful and thermal pathways in patients with diabetic neuropathy and healthy adults. This method could potentially evoke fast pain at the cutaneous nociceptors and be used to mediate nociceptive pathway of small diameter, myelinated A-fibers [11]. Research suggests that CHEPs could be utilized as an objective and non-invasive tool to detect small myelinated fiber damage in Guillain-Barré syndrome (GBS) patients.

NERVE CONDUCTION VELOCITY

The mean nerve conduction velocity (NCV) has long been a clinically valuable indicator in the diagnosis and assessment of neuromuscular disorders. The estimation of the average conduction velocity of peripheral nerves has been widely used in clinical neurology and traumatology. It has also been added to diagnostic tests of several metabolic disorders, since the myelin sheath is closely related to the propagation velocity. During a NCV test, a measurement is taken of the speed of conduction of a compound evoked potential along a nerve [12]. The measurement is taken from surface electrode patches placed on the surface of the skin to stimulate a specified nerve. An example setup for a NCV test is shown in Figure 7. One electrode stimulates the nerve while the other records when the action potential reaches its position. With this information, it can be determined how fast or slow the nerve is conducting by using the time it takes for the signal to move from the stimulating electrode to the recording electrode and the distance between the two electrodes [12].

Figure 7: Electrode configuration for nerve conduction velocity test [13]

NCV distribution estimation attempts to establish the relative number of active fibers per group of fibers. NCV tests have the potential of providing information to help assess peripheral neuropathies, but NCV tests do not provide detailed information regarding the characteristics of individual nerve fibers that contribute to the compound evoked potential. This is due to the faster conducting fibers dominating the accumulated signal that the NCV test measures. The measurement found in a NCV test is essentially created from an average of contributions made by all of the individual nerve fibers within a nerve trunk rather than investigating the individual nerve fibers themselves, so a precise diagnosis cannot always be determined solely from this test [12]. Therefore, a more useful diagnosis for a clinician would be to use a measurement technique that could extract information about the population of nerve fibers within the nerve trunk that are contributing to the evoked potential. Determining this size distribution of nerve fibers could help differentiate between various peripheral neuropathies that are shown to impact specific fiber size.

Szlavik et al. [3] presented a novel technique for estimating the size distribution of contributing nerve fibers that is linearly related to the conduction velocity distribution. The technique is based on an estimation of the group delay between two sets of recording electrodes associated with the individual fibers that contribute to a maximal compound-evoked potential. The group delay information is then used to estimate the diameters of the activated fibers as well as the propagation delays of individual single fiber-evoked potentials to a reference electrode. This process allows for reconstruction of an estimated maximal compound evoked potential, from the individual single-fiber-evoked potentials, at the first recording site. The physical setup is shown in Figure 8, where a stimulator is used to excite a subcutaneous nerve trunk consisting of a group of electrically independent nerve fibers.

Figure 8: Physical configuration for the method proposed by Szlavik et al [3]

Using a series of successively increasing current stimulus pulses, the successively recorded compound evoked potentials can be decomposed into their constituent single fiber action potentials in a manner analogous to the protocol used in the McComas et al. motor unit

number estimation technique [3, 14]. The results of the simulation study presented by Szlavik et al. demonstrate that the technique presented can, with reasonable accuracy, retrieve the conduction velocity distribution in the presence of noise introduced through variations in the perpendicular distance between the recording site and the contributing fiber. The complete methodology and results are shown in the article by Szlavik et al. [3].

EXPERIMENTAL MODEL

The rat animal model was chosen for this study to follow the Institutional Animal Care and Use Committee (IACUC) regulations on choosing the lowest-order animal model allowable. The rat was chosen due to the following reasons [15]:

- The rat is neurologically comparable to humans both anatomically and physiologically.
- The rat is of relatively small size and easily handled.
- The space around the sciatic nerve allows for ease of electrode placement.
- The rat's large sciatic nerve makes it simple to obtain a sufficient distribution of nerve fiber diameters.

The surgical protocol outlined by Sammy Nicolas [15] was followed to stimulate and extract the rat sciatic nerves used in this experiment and can be found in the dissertation: Implementation of Novel Group Delay Decomposition Method and Surgical Protocol for Assessing Peripheral Neuropathy [15].

CHAPTER III: METHODS

INTRODUCTION TO HISTOLOGY AND H&E STAINING

Histology is the science that deals with the study of tissues, and is commonly performed by examining cells and tissues by sectioning and staining [7]. Elaborate steps are taken to prepare tissue for microscopic viewing. The specimen must be fixed (preserved) and then cut into sections (slices) thin enough to transmit light or electrons. Finally the specimen must be stained in order to enhance contrast. Many dyes consist of negatively or positively charged molecules (acidic and basic stains respectively) that bind within the tissue to macromolecules of the opposite charge [16]. Because different parts of the cells and tissues take up different dyes, the stains distinguish different anatomical structures. Hematoxylin and Eosin (H&E) staining is one of the most common stains in histology and has been unchanged for many years because it works well with a variety of fixatives and displays a broad range of cytoplasmic, nuclear, and extracellular matrix features. Hematoxylin has a deep blue-purple color and stains nucleic acids by a complex, incompletely understood reaction. Eosin is pink and stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining. This method was chosen due to its simplicity and reliability for staining the nucleus and cytoplasm of tissue and for visualizing results. The histology and staining process begins with tissue extraction, tissue fixation, followed by microtomy (sectioning), deparaffinization and rehydration, staining, and microscopy for image analysis.

TISSUE EXTRACTION

The procedure for extracting the rat sciatic nerves used in this experiment was outlined in the surgical protocol written by Sammy Nicolas [15]. The dissertation outlines a surgical procedure for exposing and stimulating the sciatic nerve of an anesthetized rodent for purposes of

obtaining conduction velocity readings. This method was chosen to impose the least amount of physical trauma to the exposed nerve, in order to obtain accurate results. The nerves were previously labeled, extracted and placed in formaldehyde to preserve the tissue for future analysis.

TISSUE FIXATION

With the rat sciatic nerves extracted, it was necessary to embed the nerves in a paraffin wax block in order to support cellular components adequately during microtomy. Embedding is the casting or blocking of tissue section, which involves the enclosure of the tissue in the infiltration medium used for processing, and then allowing the medium to solidify. Paraffin wax embedding was selected due to the elasticity and density of the medium, allowing for the best tissue-medium adhesion. The tissue was embedded by placing it in an upright position in a metal mold and allowing molten paraffin wax to encase the tissue. Correct orientation was a crucial step in embedding the tissue, since incorrect placement and orientation of the tissue could result in diagnostically important tissue elements being missed or damaged during microtomy. Tubular tissues and walled specimens, such as the rat sciatic nerve, should be embedded so as to provide transverse sections showing all tissue layers. The vertically placed rat sciatic nerve was then placed on a cold block to solidify the paraffin wax encasing. The paraffin wax embedding process was repeated for three rat sciatic nerves, leaving three paraffin wax blocks labeled: 12-028 (control), 12-029 (experimental), and 12-030 (experimental). The three blocks were then stored for later analysis.

MICROTOMY

Once the tissue sections were embedded in the paraffin wax blocks, the samples would need to be sectioned to produce cross sections of the tissues for staining, imaging, and analysis. Prior to sectioning, samples were incubated at 4°C for 15 minutes to facilitate tissue sectioning and to prevent premature melting of the paraffin wax during the sectioning process. While the

samples were incubating, the tissue warm water bath was filled halfway and plugged into an outlet, the tissue oven was set to 60°C, and the microtome was set up for sectioning by placing a microtome blade in place. Once the samples had been incubated for 15 minutes, each block was placed on the tissue holder of the microtome and clamped securely. With Latex gloves, the microtome blade was placed and locked into position and the block was lowered to the level of the blade by unlocking and turning the hand wheel. The mounted blade was then moved towards the sample until it was flush against the block and locked in place. Since the actual tissue is embedded a few millimeters (mm) within the paraffin wax block, the initial section thickness was set to 15 microns (μm) and sections were taken until the tubular tissue inside the wax was exposed to the blade.

Initially, the microtome was adjusted to 6 μm and it was attempted to section the sample. Subsequent slices at 6 μm were being split upon slicing and sections were not able to be obtained. This could be due to the section thickness being too thin, the microtome blade being too dull or damaged, or the paraffin wax blocks not being cold enough. The wax block was placed in the freezer once again for incubation for an additional 15 minutes, for a total of approximately 30 minutes. After the incubation period, the blocks were removed from the freezer and steps were repeated to obtain sections at a thickness of 6 μm . Once again the sections were being split upon slicing, so the thickness of the slices was increased. The microtome was then adjusted to 7 μm and subsequent slices were taken. After many attempts, clean, undamaged 7 μm sections were produced and were carefully placed on the water surface of the warm water bath using forceps. For this step, it was important to place the sections into the water without any wrinkles, in order to facilitate the transfer from the water bath to the glass slides. Once in the water bath, adjacent strips of slices were separated to create sets of one or two sections per glass slide. To do this, forceps were used to poke the connected edges between two slices until the sections separated. The unused tissue was discarded, while the usable tissue was mounted onto a glass slide by

dipping the slide into the water bath at a 45° angle and allowing for the section to smoothly sit onto the glass slide, using forceps as a guide. During this step it was important for there not to be any air pockets, bubbles, or wrinkles in the section, in order to prevent any tissue damage during the deparaffinization, rehydration, or staining process. The slides were then placed into the microscope slide rack and allowed to dry for about 15 minutes. This procedure was followed to create multiple slides from each of the paraffin wax embedded samples. Each sample block created five slides, totaling 15 slides, with either one or two sections on each slide for the staining and imaging procedures.

DEPARAFFINIZATION AND REHYDRATION

Deparaffinization and rehydration are crucial steps in preventing poor staining of the specimen due to incomplete removal of paraffin wax or inadequate penetration of stains into the tissue. To begin this process, the slides were set in a staining rack and then placed into the slide oven, now at 60°C, for about 30 minutes, or until the paraffin wax melted and became clear. While the samples were in the oven, the following rehydration washes were set up under the fume hood: two xylene (Xylene, Richard-Allen Scientific) baths, two 100% ethanol (Sigma-Aldrich Ethanol) baths, one 95% ethanol bath, and one distilled water bath. The H & E staining washes were also set up under the fume hood during this time and included: one hematoxylin bath (Hematoxylin 7211, Richard-Allen Scientific), one distilled water bath, one bluing bath, one clarifier bath, and one Eosin bath (Eosin-Y, Richard-Allen Scientific). The washes were set up in glass trays, ensuring that enough liquid was poured in to cover the entire slide once the staining rack was dipped into the tray. Once the paraffin wax was melted, the staining rack was removed from the oven and allowed to cool for approximately five minutes. The following steps were taken to continue the deparaffinization and rehydration process: staining rack was placed in the first xylene bath for three minutes, transferred to the other xylene bath for three minutes, and then placed in the xylene bath for an additional three minutes. The staining rack was then placed in a

100% EtOH bath for two minutes, transferred to the other 100% EtOH bath for another two minutes, and then placed in the 95% EtOH bath for two minutes. The staining rack was then allowed to sit in the distilled water bath.

H&E STAINING

Once the deparaffinization and rehydration process had been completed, the samples were ready for the H & E Staining procedure. From the distilled water bath, the slide rack was placed into the Hematoxylin bath for four minutes. The rack was then transferred to a distilled water bath for one minute, then to the clarifier bath for 30 seconds, and back to the distilled water for one minute. From the distilled water, the staining rack was placed in the bluing solution for one minute, and then placed back in the distilled water for another minute. The staining rack was then placed in the 95% EtOH bath for one minute, then into the Eosin bath for one minute and 30 seconds. The staining rack was then submerged in 100% EtOH for one minute, for three repetitions. Lastly, the rack was placed in xylene for three minutes, for three repetitions, and then allowed to air dry. The Sectioning and Staining protocol that was followed during this study is included in Appendix D. Figure 9 shows the set up for the rehydration and H & E staining procedures under the fume hood.

Figure 9: View of the H & E staining procedure set-up under the fume hood.

It is crucial that during the deparaffinization, rehydration, and staining steps, fresh fluid baths are prepared before each trial to enhance the effectiveness of the stains. During an initial trial, fluid washes were reused from a previous experiment which led to undesirable results. After going through the entire staining procedure, the slides were examined under a microscope and unusable images were produced. Figure 10 shows an example of the microscopy results after old, recycled washes were used.

Figure 10: Brightfield image of an unusable H & E stained rat sciatic nerve sample using a 100x objective

An explanation for Figure 10 is that the hematoxylin stain did not penetrate into the cells, due to an error during the staining process. The hematoxylin stain should have stained the cell nuclei blue, but in this case, only the red Eosin stain can be seen. After additional similar images had been captured during microscopy analysis, it was concluded that there had been an error in the staining process, and it was to be repeated at a later time. On the next staining day, all baths were prepared on the same day as when the staining procedure was completed. The same steps as previously mentioned were repeated using the fresh fluid washes. The following steps were then taken to produce quantifiable images of the stained samples.

MICROSCOPY

Once all the slides were stained following the H&E procedure, an Olympus Laboratory Microscope (BX41, Olympus America Inc., Melville, NY) was used to visualize the results of the H&E staining process using bright field microscopy. The 100X objective (Olympus, 100x, 1.25 oil, $\infty/-$) was placed on the microscope in order to achieve the correct magnification to see the

results of the staining process. It was necessary to have an objective that could clearly see the stained nuclei and cell membrane of the corresponding tissue sample. The microscope was turned on through a series of steps and then calibrated through the live preview shown in the Q Capture Pro 6.0 software installed on the adjacent computer. First, the main power supply for the microscope and the power switch for the Q-Imaging Camera (Retiga EXi FAST 1394 digital CCD camera) installed on the microscope were turned on. Then, the lenses were switched to FW2, where the number six was selected in order to avoid a colored filter (used during fluorescent microscopy). Once the camera and microscope were turned on, the slides were prepared for viewing. Since the 100X objective required oil as the immersion fluid, a drop of immersion oil was placed onto the slide before locking the slide into the microscope stage. The objective was moved towards the slide using the focus knobs, until it touched the immersion oil, and the samples were located in the eyepiece by adjusting the focus knobs and by moving the stage. Once clear images of the samples were located in the eyepiece, images were sent to the Q Capture Pro screen by pulling out the pin located near the camera, allowing for the light to enter the camera instead of the eyepiece. Images were then captured using the Q-Imaging Camera and saved using Q Capture Pro 6.0. An example image of a correctly stained sample is shown in Figure 11. Clear images were saved for future analysis: nine images for block 12-028, six for block 12-029, and seven for block 12-030.

Figure 11: H&E stained image taken using brightfield microscopy with a 100X objective

IMAGE ANALYSIS

The images were analyzed using Image J, a Java-based image processing program commonly used for biological image analysis. This software allows for analysis of images, cell count, comparison of fiber diameters, and other measurements for each tissue sample. The primary purpose of imaging the sciatic nerve was to measure the fiber diameters. Image J allows the user to take measurements in an image using a line tool. After using the line tool, Image J outputs the measurement in units of pixels. Since pixel is not a useful unit to measure lengths in biologics, conversion from pixels to micrometers was necessary. This is easily achieved using a conversion tool in the Image J program. The conversion tool requires the user to input a known length (micrometer) of a ruler in the given image and the length in pixels of the same ruler. Measurements taken after calibration using the line tool will output lengths with the units of micrometers. In this case, the known length was the Edmund Optics Stage Micrometer (36121, 1mm/100Divisions) which was used to set the correct unit scale. Figure 12 shows the Edmund Optics Stage Micrometer. Detailed instructions on how to measure lengths in Image J is given in Appendix A.

Figure 12: Edmund Optics Stage Micrometer 1mm/100Divisions

FIBER DIAMETER MEASUREMENTS

The first step to measuring the fiber diameters was to set the appropriate scale for the images in Image J. To do this, the Edmund Optics Stage Micrometer image was opened in Image J (File → Open). In order to set the global scale, a line was drawn between a known distance and the pixel count was converted to the appropriate unit scale, using the pixel: length relationship. Since the stage micrometer is known to be 1mm/100 divisions, a line was drawn across the window (8 divisions), shown in Figure 13 , and the divisions were counted and used in the following equation:

$$1\text{mm}/100\text{ divisions} = X\text{ mm}/8\text{ divisions}$$

Figure 13: Example of setting a known distance in Image J (Line Selection tool)

The known distance between the eight divisions on the Image J window was .08 mm or 80 μm. This value was entered into the known distance box using *Analyze* → *Set Scale* and the units were set to micrometers as shown in Figure 14.

Figure 14: Example of setting the scale in Image J (*Analyze* → *Set Scale*)

Each image to be analyzed was in a window of known distance 88.74x66.30 μ m. Now that the scale was set for a known distance, it was necessary to measure each fiber diameter. This was done by clicking the *Freehand Selection* tool on the Image J toolbar and outlining the approximate circumference of each fiber shown in Figure 15.

Figure 15: Example of outlining the approximate circumference of a nerve fiber (*Freehand Selection* tool)

To transfer the data from the selection to a data window, the function *Analyze* → *Measure* (or *Ctrl M*) was used and repeated for each individual fiber diameter. These data will provide the area, mean, max, and perimeter for the selection drawn by the Freehand Tool. These data were transferred to a Microsoft Excel file by saving the data window to a location on the

computer. The area measurements and the perimeter measurements were then converted to a diameter measurement using the following equations for a circle:

$$\text{Area} = \pi d^2/4 \quad \text{or} \quad \text{Circumference} = \pi d \quad \text{where } d = \text{diameter}$$

A Microsoft Excel file was created with equations to do both of the aforementioned conversions, and an average of both conversions was used. An average was used because the fibers are not perfectly circular; therefore the diameter results using both the area equation and the circumference equation are not exact in value. Data was taken for each slide and saved onto Excel spreadsheets for use in the statistical analysis.

STATISTICAL ANALYSIS

Histograms were created for each slide in order to provide a graphical representation of the distribution of the data for the analyzed images. Histograms are useful to show the probability of distribution of a given variable by depicting the frequencies of observations occurring in certain ranges of values. Statistical analysis was done in Microsoft Office Excel due to the availability of the Histogram tool of the Analysis ToolPak. To create a histogram, the data must be organized into two columns on the worksheet, one column for input data and the other for the bin numbers. Input data is the data that you want to analyze by using the Histogram tool. Bin numbers represent intervals. The histogram tool uses the bin spacing to sort the data into respective bins. The histogram tool in Excel counts the number of data points in each data bin. A data point is included in a particular data bin if the number is greater than the lowest bound and equal to or less than the largest bound for the data bin. The output of the histogram analysis is displayed on a new tab and shows a histogram table and a column chart that reflects the data in the histogram table.

The next step was to ensure that there were two correct columns, an input variable column and a bin number column, to create the histogram. The input variable column was created from the diameter data that was calculated previously. To create the column of bin numbers, the following steps were followed:

1. The following function was used to create a count cell (Count) to get the total number of samples measured per slide:

- a. =Count(A:A) [where A is the column]

2. The following equation was used to get the approximate number of recommended bins (Bins):

- a. =(Count *2)^(1/3) [where Count is the number from the previous step]

3. To get the interval to use between bins (interval), the following equation was used:

- a. $\text{=(MAX(A:A) - MIN(A:A))/(Bins)}$ [where Bins is answer from previous step]

4. Finally, the bin column was created by taking the minimum of the input column, adding the interval value (interval) previously calculated, and repeating for subsequent values, until the number of bins (bins rounded up) was reached.

Now that there was an input column and a bin column, the Data Analysis ToolPak for a histogram was used. The **Data** tab was selected, then **Data Analysis**. In the **Data Analysis** dialog box, **Histogram** was clicked, followed by **OK**. For the **Input Range**, the input variable column was selected, and for the **Bin Range**, the Bin column was selected. Under **Output Options**, **New Worksheet Ply** was selected and renamed, and **Chart Output** was selected followed by **OK**.

Figure 16 shows the output of the Histogram ToolPak.

Figure 16: Original output using the Microsoft Office Excel ToolPak

Changes were then made to the original output in order to make the results more presentable. The changes that were made included: removing the **More** row, changing the title of the graph, changing colors of the bars on the graphs, making the bars on the graph touch to create the output shown in Figure 17 .

Figure 17: Altered output using Microsoft Office Excel ToolPak

The results from the histogram graphs were then analyzed to determine the probability of distribution for the diameters of the measured neurons.

CHAPTER IV: RESULTS

After images were captured using Q Capture Pro 6.0, the nerve fiber diameters were counted and measured using Image J software. Actual images can be found in Appendix B. The results were transferred to Microsoft Office Excel where histograms were created for each slide observed. Raw data taken from images can be found in Appendix C. Histograms were created in order to provide a graphical representation of the distribution of the data for the analyzed images. Histograms are useful to show the probability of distribution of a given variable by depicting the frequencies of observations occurring in certain ranges of values. For all of the following histograms, bin sizes were created by using the procedure found in the *Statistical Analysis* section of *Chapter III: Methods*. The y-axis of the chart will represent the frequency, while the x-axis will show the diameters of the measured neurons separated into specified bins.

Figure 18: Histogram representing data from sample 12.028.1.1

Figure 19: Histogram representing data from sample 12.028.1.2

Figure 20: Histogram representing data from sample 12.028.2.1

Figure 21: Histogram representing data from sample 12.028.2.2

Figure 22: Histogram representing data from sample 12.028.3.1

Figure 23: Histogram representing data from sample 12.028.3.2

Figure 24: Histogram representing data from sample 12.028.4.1

Figure 25: Histogram representing data from sample 12.028.5.1

Figure 26: Histogram representing data from sample 12.028.5.2

Figure 27: Histogram representing data from sample 12.029.1.1

Figure 28: Histogram representing data from sample 12.029.1.2

Figure 29: Histogram representing data from sample 12.029.2.1

Figure 30: Histogram representing data from sample 12.029.4.1

Figure 31: Histogram representing data from sample 12.029.5.1

Figure 32: Histogram representing data from sample 12.029.5.2

Figure 33: Histogram representing data from sample 12.030.1.1

Figure 34: Histogram representing data from sample 12.030.1.2

Figure 35: Histogram representing data from sample 12.030.2.1

Figure 36: Histogram representing data from sample 12.030.3.1

Figure 37: Histogram representing data from sample 12.030.3.2

Figure 38: Histogram representing data from sample 12.030.4.1

Figure 39: Histogram representing data from sample 12.030.5.1

The following histograms were created by using the information from each individual sample and combining that data to create a histogram from each tissue block. These histograms show the average diameter for every fiber measured from each sample block. These results can help differentiate whether different sample blocks had varying fiber diameters.

Figure 40: Histogram representing combined data taken from all samples from tissue block 12.028

Figure 41: Histogram representing combined data taken from all samples from tissue block 12.029

Figure 42: Histogram representing combined data taken from all samples from tissue block 12.030

CHAPTER V: DISCUSSION

The primary goal for this experiment was to acquire the results necessary to prove the efficacy of the theory previously presented by Szlavik et al. [3] for characterization of peripheral nerve fiber size distributions by group delay. The experiment presented a technique based on decomposition of the maximal compound evoked potential and subsequent determination of the group delay of the contributing nerve fibers. The fiber group delay was then utilized as an initial estimation of the nerve fiber size distribution and the associated temporal propagation delays of the single-fiber-evoked potentials to a reference electrode. The results of the simulation study demonstrated that the technique presented could, with reasonable accuracy, retrieve the fiber size distribution in the presence of recording noise for a wide range of SNR values.

In the present study, three rat sciatic nerves were extracted, embedded in paraffin wax, sectioned, stained, imaged with brightfield microscopy, and analyzed for results. One of the rat nerves served as the control nerve (12.028), while the other two nerves belonged to the experimental model chemically induced with diabetes (12.029 and 12.030). The findings from this experiment show the frequency of nerve fiber diameters for each slide analyzed using brightfield microscopy. Also, the results from each individual slide were combined to show the frequency of nerve fiber diameters per paraffin wax block.

For the paraffin wax block labeled 12.028 (control), there was a wide range of nerve fiber diameter values occurring between approximately 3 μm and 14 μm . The highest frequency (64) occurred between 9.262 μm and 10.64 μm . Although the second highest frequency occurred between 7.884 and 9.262 μm , there was also a high frequency of values occurring under 5.130 μm .

For the paraffin wax block labeled 12.029 (experimental), there was also a wide range of nerve fiber diameter values occurring between approximately 3 μm and 14 μm . The highest

frequency (58) occurred between 6.355-7.693 μm . The remaining diameters were more focused between approximately 3.5 – 9 μm ; relatively few diameters were higher than 9.031 μm .

For the paraffin wax block labeled 12.030 (experimental), there was a more expected range of results shown in the combined histogram. The most frequent diameters (190) occurred between the range of 3.237 and 3.638 μm , with a bell-shaped curve expanding outwards, with few diameters occurring after 4.440 μm .

Blocks 12.028 and 12.029 showed no trend in results, varying between smaller diameter fibers and larger diameter fibers, while block 12.030 showed a trend where most of the fiber diameter were under 4.440 μm . The control block did in fact show a wider range of values (3 μm -14 μm), while the experimental blocks generally showed a smaller diameter (< 9 μm).

The nerve fiber diameters measured for all of the combined samples ranged from approximately 2.5 μm – 14 μm . Boyd and Davey [39] published *Composition of Peripheral Nerves* which shows results that peripheral nerve fiber diameters can range from a few microns to twenty microns. The measured diameters found in this research are within range of previous publications, and are therefore realistic and can be used in future studies.

Some limitations were found during the execution of this experiment that may have led to incorrect, inconclusive, or unusable results. One of the main limitations was in the process of staining. The hematoxylin and eosin stains had been about two years expired when they were used which could have adverse effects on the staining results. Also, as mentioned before, some of the staining baths were reused, or not changed immediately before use, which could have also negatively affected the results of the staining process. There was a dramatic improvement in results when the solutions were changed prior to staining versus when solutions were reused from previous experiments. In the future, new and fresh hematoxylin, eosin, bluing solution, clarifier, xylene, and ethanol should be used to achieve the best looking images.

Some possible work for the future to improve results would be to change the method of embedding and staining. The gold standard protocol for processing peripheral nerve tissue to obtain high resolution imaging of myelinated fibers is resin embedding after 2% osmium tetroxide postfixation [17]. The advantages of staining with osmium tetroxide before embedding are: (1) it prevents myelin sheath swelling, which usually occurs during paraffin embedding, thanks to its action as a lipid fixative; (2) it provides the typical dark and sharp myelin stain, which greatly facilitates the identification of nerve fibers. Measuring the myelin sheath would help discriminate between myelinated and unmyelinated fibers, which would have been helpful when determining which type of peripheral nerve fibers are being affected. Another method to detect the myelin sheaths more clearly would be to use Masson's trichrome counterstaining, which in particular permits clear imaging of the nerve's connective structures [17].

In conclusion, the goal of this study was to create data to be used in an algorithm to prove the efficacy of a novel method of characterizing peripheral nerve fiber distributions by group delay. The results found during execution of this experiment will be used to support continuing research for a novel method to characterize peripheral neuropathies using group delay.

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APPENDIX A – IMAGE J BASIC INSTRUCTIONS [38]

ImageJ Basics

(Version 1.38)

ImageJ is an image analysis program that was created at the National Institutes of Health. It is in the public domain and runs on a variety of operating systems. You may download this program from the source (<http://rsb.info.nih.gov/ij/>). The ImageJ website has instructions for use of the program and links to useful resources.

Installing ImageJ on your PC (Windows operating system): Copy the ImageJ folder and transfer it to the C drive of your personal computer. Open the ImageJ folder in the C drive and copy the shortcut (microscope with arrow) to your computer's desktop. Double click on this desktop shortcut to run ImageJ. See the ImageJ website for Macintosh instructions.

ImageJ Window: The ImageJ window will appear on the desktop. Note that this window has a Menu Bar, a Tool Bar and a Status Bar.

Adjusting Memory Allocation: Use the *Edit* → *Options* → *Memory* command to adjust the default memory allocation. Setting the maximum memory value to more than about 75% of real RAM may result in poor performance due to virtual memory paging.

Opening an Image File: Select *File* → *Open* from the menu bar to open a stored image file.

Tool Bar: The various buttons on the tool bar allow you measure, draw, label, fill, etc. A right-click or a double left-click may expand your options with some of the tool buttons.

Area Selection Tools: The first four buttons on the tool bar allow you to surround an area on the image with a rectangle, oval, polygon or freehand shape. After selection, these areas may be altered, analyzed, copied, etc. using the menu commands. The status bar, below the tool bar, gives information such as the coordinates (xx, yy) of the selection on the frame.

Line Selection Tools: This button allows you to create straight, segmented or freehand lines (right-click to select line type). Information about the line is displayed on the status bar.

Double-click on the button to alter the line width, select *Analyze* → *Measure* (or Ctrl+M) to record a line length and *Edit* → *Draw* (or Ctrl+D) to make a line permanent.

Angle Tool: Draws two intersecting lines and measures the formed angle.

Point Tool: When 'Auto-Measure' is selected, this tool allows you to mark locations on an

image; with each click the coordinates of the mark (xx, yy) and brightness values (0-255) are recorded in a data window. Color images will have three brightness readings displayed on the status bar, one each for the red, green and blue channels, however only a single grayscale brightness value will appear in the data window.

Wand Tool: This tool automatically finds the edge of an object and traces its shape. It works best with high contrast images. Place the wand to the left of an edge and click. The algorithm will search to the right for an edge. It will then trace along the edge of the object until it returns to the starting point.

Text Tool: Double click on this button to select a font and size. Single click the button, click-drag a text box and type the label. Move the box to the desired location and permanently set the text in place with *Edit* → *Draw* (or Ctrl+D).

Magnifying Glass: Left-click on the image to magnify; right-click to reduce the image size.

Scrolling Tool: This button allows you to move an image if it is larger than the window.

Color Picker: This tool sets the foreground drawing/text color when a color on an image is 'picked up' with the eyedropper. Colors also may be selected from the *Colors* window by double-clicking the color picker button (or via *Image* → *Color* → *Color Picker*). Use Alt+click to change the background color. The icon for this tool (eye dropper) shows the current foreground color while the frame around it shows the background color.

Other Tools: The remaining tool buttons are similar to those found in drawing programs (spray can, flood fill, etc.) and can be easily used after a bit of experimentation.

Image Editing and Analysis

The following is a small sampling of processing techniques that are possible with ImageJ. It is common to use ImageJ in conjunction with a photo editing program such as Adobe Photoshop. During analysis, measured values can be easily transferred to a spreadsheet with a 'cut and paste'. Refer to the ImageJ and NIH Image websites for more information

Undo. **Edit** → **Undo** reverses the preceding action. Only one back-step is possible.

Revert. **File** → **Revert** converts all changes back to the original saved version.

Cropping. Surround the area with the rectangular selection tool followed by **Image** → **Crop**.

Clear Outside. Make a perimeter with an area selection tool followed by **Edit** → **Clear Outside**.

This technique is useful for clearing extraneous objects near an area of interest. **Edit** → **Clear** clears inside of the perimeter.

Enhancing Brightness and Contrast. **Image** → **Adjust** → **Brightness/Contrast**; click 'Auto' or set manually with the sliders. Also try **Process** → **Enhance Contrast**.

Removing Noise. **Process** → **Noise** → **Despeckle** or try **Process** → **Filters** → **Median**

Rotating an Image. **Image** → **Rotate** and select type of rotation

Converting to Grayscale. **Image** → **Type** → **8-bit** converts the image to 256 shades (8-bit) of gray. In this scale 0 = pure black and 255 = pure white.... a grayscale reading of 128 would be a medium gray.

Thresholding (Binary Contrast Enhancement). This is commonly used when detecting edges, counting particles or measuring areas. A grayscale image is converted to binary (a.k.a. halftone or black & white) by defining a grayscale cutoff point. Grayscale values below the cutoff become black and those above become white. The procedure: First convert the image to 8-bit grayscale as described above (**Image** → **Type** → **8-bit**). Create a 'thresholded' binary image by selecting **Process** → **Binary** → **Make Binary**. A less automated procedure involves: **Image** → **Adjust** → **Threshold**; use the slider to adjust the threshold. The red areas will become the black portions in the binary image. Click 'Apply' to complete the conversion. 'Brightness slicing' is a similar procedure that uses both upper and lower threshold values.

Measuring and Counting Objects

Setting Measurement Scale. Draw a line between two points of known distance such as a ruler on the photograph. Go to **Analyze** → **Set Scale**. In the *Set Scale* window the length of the line, in

pixels, will be displayed. Type the known distance and units of measure in the appropriate boxes and click OK. Measurements will now be shown using these settings. If the pixel:length relationship is known from a previous measurement you may directly type this information in the *Set Scale* window. Check 'global' to apply this scale to other image frames.

Set Measurements. Choose parameters to be measured via *Analyze* → *Set Measurements*.

Measuring Distance Between Points. Draw a line between two points. The status bar will show the angle (from horizontal) and the length. *Analyze* → *Measure* (or Ctrl+M or simply type M on the keyboard) transfers the values to a data window.

Measuring Area. Surround an area with a perimeter. This can be done with an area selection tool, the wand (for high contrast images) or with *Analyze Particles* (see below). *Analyze* → *Measure* (or keyboard M) transfers the area measurement to a data window.

Counting Particles. As described above, convert the image to 8-bit grayscale and then 'threshold' the image. Go to *Analyze* → *Analyze Particles*, type the upper and lower limits for the particle size, toggle 'show outlines' and check 'Display Results'. Click OK and each counted particle will be outlined and numbered in a new window (numbers may be very small). The data window contains measurements for each particle.

Saving Files. Images from digital cameras are usually saved as JPEG files. JPEG is a type of memory compression that results in the loss of some data. A JPEG image degrades each time it is opened, edited and resaved. It is best to save a file in a 'lossless' format such as a TIFF during the editing process (*File* → *Save As* → *Tiff*).

Printing. Should you encounter printing problems, save the processed image and print with a photo editor. Saved images also may be inserted into MS Word for printing.

APPENDIX B – H & E STAINED IMAGES

Figure 43: Block 12-028, Slide 1, A

Figure 44: Block 12-028, Slide 1, B

Figure 45: Block 12-028, Slide 2, A:

Figure 46: Block 12-028, Slide 2, B

Figure 47: Block 12-028, Slide 3, A

Figure 48: Block 12-028, Slide 3, B

Figure 49: Block 12-028, Slide 4, A

Figure 50: Block 12-028, Slide 5, A

Figure 51: Block 12-028, Slide 5, B

Figure 52: Block 12-029, Slide 1, A

Figure 53: Block 12-029, Slide 1, B

Figure 54: Block 12-029, Slide 2, A

Figure 55: Block 12-029, Slide 4, A

Figure 56: Block 12-029, Slide 5, A

Figure 57: Block 12-029, Slide 5, B

Figure 58: Block 12-030, Slide 1, A

Figure 59: Block 12-030, Slide 1, B

Figure 60: Block 12-030, Slide 2, A

Figure 61: Block 12-030, Slide 3, A

Figure 62: Block 12-030, Slide 3, B

Figure 63: Block 12-030, Slide 4, A

Figure 64: Block 12-030, Slide 5, A

APPENDIX C – RAW MEASUREMENT DATA

Sample: 12.028.1.1

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	79.8541	81.903	60	110	33.6022	10.08332276	10.69591246	10.38962
2	85.5443	82.2456	59	118	33.9566	10.43639716	10.80872148	10.62256
3	90.0802	85.6158	63	120	35.4999	10.70951319	11.29996913	11.00474
4	77.2366	82.0626	58	114	31.7468	9.916687623	10.10532029	10.011
5	73.7737	83.6054	57	108	32.011	9.69183121	10.18941777	9.940624
6	86.0117	75.5752	57	116	34.2915	10.4648697	10.91532346	10.6901
7	64.901	86.4719	61	109	29.8751	9.090353112	9.509539681	9.299946
8	73.9322	82.744	62	116	33.2643	9.70223689	10.58835555	10.1453
9	66.1569	85.0985	61	107	29.8197	9.177885445	9.491905313	9.334895
10	61.8039	85.5589	65	116	30.1617	8.870804332	9.600767294	9.235786
11	88.0439	85.3444	61	117	34.5828	10.5877748	11.00804713	10.79791
12	66.9292	82.4189	47	120	30.2748	9.231300241	9.636768142	9.434034
13	95.1974	84.0054	56	111	36.4994	11.00950018	11.61811986	11.31381
14	56.0649	83.2074	61	105	27.401	8.44890808	8.722009191	8.585459
15	20.1109	81.002	57	104	16.8972	5.060236473	5.378545809	5.219391
16	40.4006	80.6848	49	111	24.1462	7.172143442	7.685974174	7.429059
17	36.7222	84.8393	60	111	24.0899	6.837847411	7.668053327	7.25295
18	79.1794	85.8157	59	114	34.3363	10.0406346	10.92958374	10.48511
19	56.5526	83.5753	62	109	28.2064	8.48557639	8.978375974	8.731976
20	84.5363	87.2615	59	115	36.1144	10.37472699	11.49557055	10.93515
21	42.8921	78.9782	50	115	25.2809	7.389987678	8.047160402	7.718574
22	66.3927	83.9333	59	117	30.4613	9.194227054	9.696132936	9.44518
23	52.3297	81.4141	62	114	26.699	8.162612535	8.498555651	8.330584
24	58.4589	82.3681	56	114	28.1462	8.627408836	8.959213719	8.793311
25	52.4923	83.916	58	120	27.0442	8.175284225	8.608436224	8.39186
26	85.7272	86.8515	65	116	34.2283	10.44754809	10.89520628	10.67138
27	55.0122	81.7395	60	112	27.1527	8.369211939	8.642972847	8.506092

Sample: 12.028.1.2

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	82.6951	85.3681	53	127	33.2392	10.26112	10.58037	10.42075
2	61.2146	88.7193	61	124	28.5249	8.828411	9.079758	8.954085
3	16.2943	85.1462	68	105	14.9982	4.554838	4.774075	4.664457
4	42.2336	84.005	55	112	23.952	7.333041	7.624158	7.4786
5	66.6203	88.8733	63	132	29.6858	9.209973	9.449284	9.329628
6	53.423	84.5133	59	134	28.2725	8.247441	8.999416	8.623428
7	58.4589	86.7697	62	122	28.1486	8.627409	8.959978	8.793693
8	76.0742	84.6863	54	129	32.8502	9.841782	10.45654	10.14916
9	90.9622	89.5311	61	132	36.1242	10.76182	11.49869	11.13025
10	64.901	88.5487	58	134	30.3436	9.090353	9.658668	9.37451
11	38.6203	85.7664	56	124	23.3002	7.012339	7.416684	7.214511
12	39.3722	83.6652	62	129	25.4654	7.080271	8.105889	7.59308
13	91.0191	89.5551	52	133	35.4424	10.76518	11.28167	11.02342
14	36.9092	86.556	62	126	22.1036	6.855235	7.035794	6.945515
15	32.2473	85.6288	56	123	21.5432	6.407694	6.857414	6.632554
16	66.7747	89.3208	62	135	30.2875	9.220639	9.640811	9.430725
17	66.7869	84.7482	59	123	29.8912	9.221482	9.514664	9.368073
18	39.0959	85.7504	59	122	22.8958	7.055384	7.287959	7.171672
19	86.8693	85.3448	56	143	35.4597	10.51691	11.28717	10.90204
20	95.3437	87.6245	55	129	35.6277	11.01796	11.34065	11.1793
21	69.376	86.968	51	125	30.1809	9.398525	9.606879	9.502702
22	65.1205	89.6056	58	133	29.5944	9.105712	9.42019	9.262951
23	45.5339	84.4758	60	120	24.5802	7.614169	7.824121	7.719145
24	42.1117	93.8546	55	130	23.6087	7.322451	7.514883	7.418667
25	84.6826	91.9836	56	127	35.2202	10.3837	11.21094	10.79732
26	89.4787	85.1524	53	143	35.1643	10.6737	11.19314	10.93342
27	131.1351	86.1811	55	133	41.9901	12.92155	13.36586	13.14371
28	75.0133	90.7622	61	139	31.3901	9.772917	9.991779	9.882348
29	87.5684	86.4981	63	129	35.8386	10.55915	11.40778	10.98346
30	72.7413	87.5652	54	129	31.658	9.623778	10.07705	9.850416
31	61.5641	86.4639	60	125	28.8804	8.853578	9.192917	9.023248

Sample: 12.028.2.1

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	9.1531	75.8606	52	95	11.2497	3.413809	3.580891	3.49735
2	10.3521	74.3632	52	98	12.4643	3.630524	3.96751	3.799017
3	12.8762	81.7639	53	106	14.1785	4.049011	4.513157	4.281084
4	9.9579	72.9286	56	92	12.1602	3.560729	3.870712	3.715721
5	10.7423	75.3299	52	95	12.3844	3.698313	3.942077	3.820195
6	7.3079	74.2603	49	98	9.8995	3.050362	3.151109	3.100735
7	8.3565	77.1342	57	95	10.7397	3.261875	3.418553	3.340214
8	10.1896	67.8205	45	94	11.8096	3.601916	3.759112	3.680514
9	9.4498	70.0662	50	90	11.5045	3.468697	3.661996	3.565347
10	10.7748	60.4738	45	85	12.3441	3.703904	3.929249	3.816576
11	9.5758	67.5497	50	98	11.4437	3.491746	3.642643	3.567194
12	15.7538	53.5106	32	85	14.478	4.478656	4.608491	4.543573
13	10.8155	68.6659	49	87	11.9653	3.710892	3.808673	3.759783
14	9.7912	73.5509	52	91	11.7393	3.530799	3.736735	3.633767
15	7.2875	72.4138	55	90	10.1376	3.046101	3.226898	3.1365
16	8.7467	72.4266	58	86	11.1619	3.337161	3.552943	3.445052
17	13.4777	72.7979	54	95	13.733	4.142504	4.37135	4.256927
18	12.108	68.4371	54	80	12.6201	3.92637	4.017103	3.971736
19	11.7422	62.3752	51	85	12.8058	3.866605	4.076213	3.971409
20	10.9659	61.5226	49	80	12.2546	3.736605	3.90076	3.818683
21	7.2632	64.1147	49	102	10.3211	3.041018	3.285308	3.163163
22	8.0964	66.9563	48	86	10.6608	3.21071	3.393438	3.302074
23	9.7912	57.9543	47	78	11.4111	3.530799	3.632266	3.581533
24	10.157	57.3093	36	78	11.9309	3.59615	3.797723	3.696937
25	12.3112	55.2595	42	73	12.9668	3.95918	4.127461	4.04332
26	14.4613	37.0149	23	52	14.5856	4.291002	4.642741	4.466871
27	8.9336	51.7152	32	68	11.2547	3.372627	3.582482	3.477555
28	8.7711	47.336	35	62	11.2672	3.341813	3.586461	3.464137

Sample: 12.028.2.2

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	15.1197	73.5774	56	100	14.4917	4.387596	4.612851	4.500224
2	13.4127	77.8261	54	98	13.6337	4.132503	4.339741	4.236122
3	10.5635	78.9681	63	98	12.3675	3.667406	3.936698	3.802052
4	9.1287	68.89	47	96	11.2437	3.409255	3.578981	3.494118
5	12.4413	72.3976	54	102	13.2001	3.980045	4.201722	4.090883
6	9.3604	77.3752	63	95	11.2595	3.45225	3.58401	3.51813
7	9.6937	79.0122	55	112	11.6299	3.513176	3.701912	3.607544
8	11.3073	80.5482	59	112	12.303	3.794325	3.916167	3.855246
9	10.279	81.9518	61	109	11.6733	3.617683	3.715727	3.666705
10	10.9862	78.5797	57	100	12.6091	3.740062	4.013601	3.876832
11	13.6321	55.2976	35	123	14.3143	4.166165	4.556383	4.361274
12	13.185	70.7028	52	88	13.6493	4.097275	4.344707	4.220991
13	15.957	69.8136	56	84	14.8239	4.507448	4.718594	4.613021
14	12.3925	70.8285	48	89	13.3335	3.972231	4.244185	4.108208
15	14.693	74.1071	50	98	15.3799	4.325241	4.895574	4.610408
16	8.3849	79.5313	62	112	10.6917	3.267413	3.403274	3.335343
17	11.3276	53.8159	35	104	12.4754	3.797729	3.971043	3.884386
18	7.9907	71.6424	50	92	10.611	3.189683	3.377586	3.283634
19	8.0313	71.6397	50	98	10.6967	3.197776	3.404865	3.30132
20	6.1698	84.334	60	102	9.2294	2.80279	2.937809	2.8703
21	4.288	80.5592	61	99	8.0499	2.33659	2.562363	2.449476
22	13.185	75.9069	53	99	13.4501	4.097275	4.2813	4.189287
23	11.5227	60.4469	36	139	12.4976	3.830295	3.97811	3.904202
24	10.913	75.1918	51	101	12.3252	3.727581	3.923233	3.825407
25	11.3195	57.47	30	111	12.4739	3.796371	3.970566	3.883468
26	12.8518	70.3327	48	89	13.9253	4.045172	4.432561	4.238867

Sample: 12.028.3.1

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	78.8095	115.4305	77	163	33.1928	10.01715	10.5656	10.29138
2	38.4618	115.0361	82	158	22.6314	6.997934	7.203798	7.100866
3	45.0747	103.2017	70	139	25.8512	7.575678	8.228693	7.902185
4	43.5668	104.1046	70	129	23.8972	7.447884	7.606715	7.527299
5	70.7782	111.4082	78	152	31.9577	9.493029	10.17245	9.832741
6	79.0859	110.7625	85	144	33.928	10.0347	10.79962	10.41716
7	94.9372	102.2321	69	139	35.7057	10.99444	11.36548	11.17996
8	132.6715	66.3828	39	120	42.5033	12.99702	13.52922	13.26312
9	79.0859	109.4768	81	176	32.455	10.0347	10.33075	10.18273
10	75.3263	115.4132	83	160	32.2356	9.793285	10.26091	10.0271
11	90.2306	112.1494	72	155	34.5312	10.71845	10.99162	10.85504
12	75.2328	103.2489	68	145	32.7053	9.787205	10.41042	10.09881
13	71.9244	107.5847	73	143	30.9184	9.569587	9.841632	9.70561
14	112.5972	115.6456	79	158	38.5555	11.97344	12.2726	12.12302
15	105.7283	118.5683	78	171	39.3802	11.60248	12.53511	12.06879
16	44.6276	96.3694	59	147	25.8693	7.538012	8.234454	7.886233
17	82.4635	115.755	70	155	34.1534	10.24675	10.87136	10.55906
18	81.1832	102.9145	68	145	33.0047	10.16689	10.50572	10.33631
19	100.6356	111.3244	72	155	36.331	11.31959	11.56452	11.44206
20	90.8078	98.4906	45	151	34.6377	10.75268	11.02552	10.8891
21	72.8917	116.9795	78	162	30.9981	9.633722	9.867002	9.750362
22	94.8234	115.0727	81	158	35.1277	10.98785	11.18149	11.08467
23	33.8162	113.352	69	146	21.5168	6.561716	6.84901	6.705363
24	55.4796	115.2544	83	147	27.4648	8.40469	8.742317	8.573504
25	50.9762	116.1447	86	157	26.8069	8.056359	8.532901	8.29463
26	68.5956	121.5901	84	153	30.5755	9.345514	9.732484	9.538999
27	60.8366	128.3787	96	159	29.8039	8.801112	9.486876	9.143994
28	62.9867	116.7661	86	157	29.1436	8.955287	9.276696	9.115991

Sample: 12.028.3.1

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	82.9837	119.1332	85	190	33.2651	10.27901	10.58861	10.43381
2	80.5979	117.9704	86	158	32.3038	10.13017	10.28262	10.2064
3	83.3454	114.3326	81	163	34.7892	10.30139	11.07375	10.68757
4	59.7676	111.0705	81	142	28.3655	8.723444	9.029019	8.876231
5	49.765	112.5706	85	148	26.6579	7.960073	8.485473	8.222773
6	83.3089	116.4158	79	158	32.9622	10.29914	10.49219	10.39566
7	47.8425	115.88	84	148	26.4088	7.804804	8.406182	8.105493
8	67.2909	117.6669	89	155	30.1732	9.256211	9.604428	9.430319
9	64.393	122.8082	79	152	29.2033	9.054707	9.295699	9.175203
10	80.0045	121.0675	79	170	32.8741	10.09281	10.46415	10.27848
11	73.0096	114.427	82	153	31.2584	9.64151	9.949858	9.795684
12	63.072	118.6081	75	167	30.0942	8.961348	9.579281	9.270315
13	52.6223	122.4705	83	157	27.4121	8.185401	8.725542	8.455472
14	75.1718	122.7157	88	160	31.5978	9.783236	10.05789	9.920564
15	60.231	119.441	79	152	29.7574	8.757197	9.472075	9.114636
16	61.4991	120.8784	83	157	29.0751	8.848903	9.254892	9.051897
17	58.8165	117.5038	83	153	28.7013	8.653756	9.135908	8.894832
18	61.6332	124.3264	86	158	28.3229	8.858545	9.015459	8.937002
19	62.7956	112.1819	82	147	28.6738	8.941691	9.127154	9.034423
20	48.2043	124.4726	91	162	25.0028	7.834259	7.958638	7.896449
21	57.1339	118.9985	87	162	27.4376	8.529076	8.733659	8.631368
22	51.6103	114.8006	83	154	26.2104	8.106311	8.343029	8.22467
23	48.1677	122.676	83	157	25.3311	7.831285	8.06314	7.947212
24	92.5758	111.2137	84	151	34.9831	10.85685	11.13547	10.99616
25	73.229	102.5956	69	136	32.4723	9.655986	10.33625	9.99612

Sample: 12.028.4.1

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	83.8616	116.2334	80	154	33.7197	10.33324	10.73331	10.53328
2	99.2984	121.3361	86	154	36.4578	11.24414	11.60488	11.42451
3	78.5331	119.455	94	151	32.3218	9.999572	10.28835	10.14396
4	94.7421	117.7187	83	150	35.5558	10.98314	11.31776	11.15045
5	83.4714	120.3775	86	171	33.5885	10.30917	10.69155	10.50036
6	56.5608	120.6223	83	158	27.835	8.486192	8.860156	8.673174
7	71.1602	114.6326	80	157	30.3356	9.518612	9.656121	9.587367
8	87.479	113.006	77	151	35.0945	10.55375	11.17093	10.86234
9	60.6699	113.7706	84	149	28.7155	8.789045	9.140428	8.964736
10	65.5107	110.4944	78	146	31.3907	9.132952	9.99197	9.562461
11	67.1283	125.2678	82	163	30.2489	9.245021	9.628524	9.436772
12	58.3207	121.2369	91	153	27.75	8.617205	8.833099	8.725152
13	69.0833	115.5393	88	147	30.1948	9.378677	9.611303	9.49499
14	67.2137	120.8933	91	157	29.5263	9.250899	9.398513	9.324706
15	78.4193	114.4106	81	149	32.2365	9.992325	10.2612	10.12676
16	110.7804	115.2216	82	170	38.8322	11.87645	12.36067	12.11856
17	95.681	113.9726	74	160	35.8703	11.03743	11.41787	11.22765
18	35.5192	107.3832	74	149	22.1414	6.724913	7.047827	6.88637
19	74.485	116.7521	78	168	31.8	9.738442	10.12225	9.930348
20	56.0121	114.0788	79	143	28.2664	8.444929	8.997475	8.721202
21	80.6629	112.3432	77	155	33.5586	10.13426	10.68203	10.40815
22	114.7433	123.3415	76	158	39.6285	12.08701	12.61414	12.35057
23	49.1594	114.4448	78	147	26.8285	7.911491	8.539777	8.225634
24	45.8388	116.8012	93	144	25.5751	7.639619	8.140807	7.890213
25	60.2838	115.745	77	171	28.1757	8.761034	8.968604	8.864819
26	54.3009	89.3909	49	131	27.3165	8.31493	8.695112	8.505021
27	46.1964	118.3216	76	159	25.8109	7.66936	8.215865	7.942612

Sample: 12.028.5.1

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	10.7301	166.4178	138	194	12.1041	3.696213	3.852855	3.774534
2	6.4543	140.8659	105	160	9.5039	2.866683	3.025185	2.945934
3	7.6046	166.6526	106	194	10.711	3.111668	3.409417	3.260543
4	12.9615	136.4785	93	176	13.7336	4.0624	4.371541	4.21697
5	7.95	162.9755	103	193	11.0806	3.181549	3.527065	3.354307
6	10.157	140.0088	95	186	11.9902	3.59615	3.816599	3.706375
7	12.8111	130.3912	86	174	13.1551	4.038762	4.187398	4.11308
8	14.6401	147.2282	112	166	14.2951	4.317448	4.550272	4.43386
9	9.4823	157.1569	125	185	11.8349	3.474657	3.767166	3.620911
10	11.7178	142.2345	98	187	12.8029	3.862585	4.07529	3.968938
11	7.4501	132.5963	81	179	10.0034	3.079896	3.184181	3.132039
12	10.9699	135.2864	88	172	12.0278	3.737287	3.828568	3.782927
13	7.9582	127.2513	64	192	10.5176	3.183189	3.347856	3.265523
14	7.0681	126.13	70	160	9.8764	2.999897	3.143756	3.071827
15	6.4015	124.1511	81	157	9.3807	2.854933	2.98597	2.920451
16	7.125	98.4997	63	157	10.4284	3.011948	3.319463	3.165705
17	12.8477	150.702	113	182	13.1254	4.044527	4.177945	4.111236
18	9.458	140.8483	92	176	11.6671	3.470202	3.713753	3.591977
19	10.0961	135.0612	76	175	12.3584	3.585353	3.933801	3.759577
20	6.5031	105.8962	68	143	10.4971	2.8775	3.341331	3.109415
21	8.9214	137	102	174	11.5062	3.370323	3.662537	3.51643
22	3.5808	107.9092	69	156	8.2135	2.135232	2.614438	2.374835
23	11.2707	121.9174	74	166	12.4144	3.788179	3.951626	3.869903
24	7.6249	154.5949	107	178	10.4707	3.115818	3.332927	3.224373
25	8.9296	160.1124	117	186	11.1139	3.371872	3.537664	3.454768
26	6.6128	116.5605	80	156	10.0138	2.901668	3.187492	3.04458
27	7.6005	136.6989	104	175	10.3692	3.110829	3.300619	3.205724
28	15.7456	155.0534	118	195	15.2878	4.47749	4.866258	4.671874

Sample: 12.028.5.2

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	73.2575	118.3002	72	191	30.8987	9.657864	9.835362	9.746613
2	61.5925	122.9098	77	186	28.9411	8.85562	9.212238	9.033929
3	40.3843	117.9231	83	173	23.9943	7.170696	7.637623	7.40416
4	69.7905	120.7892	76	168	30.6248	9.42656	9.748177	9.587368
5	72.3593	121.7994	82	172	30.6383	9.598475	9.752474	9.675474
6	62.3932	122.5856	78	171	29.1292	8.912996	9.272112	9.092554
7	85.9182	122.4486	74	175	34.8842	10.45918	11.10399	10.78158
8	78.7404	121.4291	79	170	32.379	10.01276	10.30656	10.15966
9	75.5498	121.7657	83	161	31.6776	9.807803	10.08329	9.945548
10	70.9855	123.2955	77	186	31.0544	9.506921	9.884923	9.695922
11	59.1458	116.4623	78	176	28.1821	8.677947	8.970641	8.824294
12	50.0455	122.2662	81	179	26.1897	7.982475	8.33644	8.159458
13	87.5074	125.957	82	177	34.4786	10.55547	10.97488	10.76517
14	75.4848	123.3844	83	180	31.4624	9.803583	10.01479	9.909188
15	55.1138	125.3476	87	166	27.0925	8.376937	8.623811	8.500374
16	85.0078	128.9011	81	184	34.0847	10.40362	10.8495	10.62656
17	101.0623	123.0127	83	167	37.9439	11.34357	12.07792	11.71074
18	89.4746	131.6503	77	184	35.6292	10.67345	11.34113	11.00729
19	82.4228	124.7247	82	170	33.9575	10.24422	10.80901	10.52661
20	79.0615	120.6226	80	163	33.2376	10.03316	10.57986	10.30651
21	61.4462	121.4785	78	164	28.2943	8.845096	9.006355	8.925726
22	77.5374	129.8218	77	184	35.536	9.935979	11.31146	10.62372
23	116.6373	135.5021	86	182	40.3713	12.18635	12.85058	12.51847
24	59.3612	125.9423	83	167	28.5858	8.693735	9.099143	8.896439
25	69.6564	134.6757	87	191	30.3849	9.417499	9.671814	9.544656
26	81.74	127.3734	81	175	33.538	10.2017	10.67548	10.43859
27	64.2629	114.4749	77	166	29.0143	9.045555	9.235539	9.140547

Sample: 12.029.1.1

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	74.8345	123.9549	92	163	32.5892	9.761262	10.37346	10.06736
2	87.9952	135.7201	92	175	34.3988	10.58485	10.94948	10.76716
3	94.2544	131.6187	87	184	36.9454	10.95484	11.76009	11.35746
	102.005							
4	3	129.1601	84	186	36.6626	11.39637	11.67007	11.53322
5	81.3092	132.1095	91	173	35.5777	10.17478	11.32473	10.74976
6	74.6475	131.8298	91	170	31.943	9.749059	10.16777	9.958416
7	38.2586	121.4103	84	154	22.8766	6.979424	7.281848	7.130636
8	92.397	124.9366	83	184	35.6083	10.84636	11.33447	11.09042
9	59.5359	101.7781	39	164	28.4943	8.706518	9.070017	8.888268
10	86.154	127.2589	93	170	35.2404	10.47352	11.21737	10.84545
	121.364							
11	2	93.1262	53	155	40.6514	12.43084	12.93974	12.68529
12	78.4071	127.3054	83	171	32.4195	9.991547	10.31945	10.1555
	101.720							
13	8	126.9202	82	159	36.3074	11.38046	11.557	11.46873
14	48.2571	125.4566	89	159	25.579	7.838549	8.142049	7.990299
15	74.3143	128.1488	81	183	31.6756	9.727276	10.08266	9.904966
16	68.0388	122.0328	82	162	30.2147	9.307507	9.617638	9.462572
17	99.4813	125.5475	87	172	37.2907	11.25449	11.87	11.56224
18	56.0934	122.3871	84	186	26.9554	8.451055	8.58017	8.515613
19	67.6567	112.6466	73	172	30.0162	9.281335	9.554453	9.417894
20	38.3643	110.1416	49	153	22.2733	6.989059	7.089812	7.039435
21	40.1038	116.4153	83	153	22.9872	7.14575	7.317053	7.231402
22	98.2335	121.6534	78	173	42.7112	11.18368	13.5954	12.38954
23	57.8411	113.3646	63	178	27.6743	8.5817	8.809003	8.695352
24	98.1807	129.1536	80	180	38.2244	11.18068	12.1672	11.67394
25	66.6812	131.1824	84	188	30.094	9.214182	9.579218	9.3967

Sample: 12.029.1.2

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	8.8076	112.2713	93	132	12.4462	3.348759	3.961749	3.655254
2	8.8605	113.639	90	134	12.1998	3.3588	3.883317	3.621059
3	11.7991	110.3317	84	137	13.4472	3.875962	4.280377	4.078169
4	5.6252	112.0253	87	139	9.3141	2.676234	2.96477	2.820502
5	9.4132	116.12	91	147	12.5026	3.461973	3.979701	3.720837
6	8.3443	110.6873	85	133	11.664	3.259493	3.712767	3.48613
7	10.4253	120.593	91	141	12.4423	3.643337	3.960507	3.801922
8	10.661	111.7934	76	133	14.1366	3.684292	4.49982	4.092056
9	4.7473	81.0933	55	137	9.2711	2.458546	2.951083	2.704815
10	12.1608	110.7604	85	134	13.4011	3.934922	4.265703	4.100312
11	16.5545	109.768	89	135	16.9793	4.591061	5.404679	4.99787
12	12.4941	115.3243	93	146	13.2075	3.988481	4.204078	4.09628
13	9.4092	105.4799	87	132	12.0845	3.461238	3.846616	3.653927
14	15.0953	110.2092	73	145	15.7291	4.384054	5.006728	4.695391
15	10.0107	72.5489	43	124	12.3494	3.570157	3.930936	3.750546
16	13.7012	107.8529	76	152	14.475	4.17671	4.607536	4.392123
17	16.7049	108.7311	81	132	14.9493	4.611869	4.75851	4.68519
18	8.4012	104.3638	69	137	11.6491	3.270587	3.708024	3.489305
19	17.0178	103.2837	74	134	15.7037	4.654862	4.998643	4.826752
20	6.9258	108.0839	79	154	9.9594	2.969546	3.170175	3.069861
21	9.1328	102.4424	79	128	11.5795	3.410021	3.685869	3.547945
22	9.8278	105.0054	79	136	11.9646	3.537392	3.80845	3.672921
23	8.8564	105.4016	77	148	13.1326	3.358023	4.180236	3.76913
24	7.442	107.4014	91	131	10.9515	3.078222	3.485971	3.282096
25	11.9617	96.6174	74	119	13.433	3.902577	4.275857	4.089217
26	10.9903	111.453	80	149	12.4679	3.74076	3.968656	3.854708
27	16.8512	117.4086	67	148	15.5755	4.632021	4.957836	4.794928
28	6.7348	103.2227	76	121	9.7507	2.928312	3.103744	3.016028
29	7.0112	99.2151	80	127	9.9929	2.987798	3.180839	3.084318
30	6.938	106.3357	76	131	9.6927	2.97216	3.085282	3.028721
31	5.2797	108.6266	79	131	8.3428	2.592744	2.655596	2.62417
32	18.6436	103.5335	68	144	17.152	4.872142	5.459651	5.165897
33	11.2504	99.4057	71	138	12.5906	3.784766	4.007712	3.896239
34	9.3076	103.9921	74	136	11.5204	3.4425	3.667057	3.554778
35	8.9743	108.1159	79	138	13.5181	3.380301	4.302945	3.841623
36	8.4784	99.4492	73	124	11.3006	3.28558	3.597093	3.441336
37	13.1972	112.8164	79	158	13.931	4.09917	4.434375	4.266773
38	17.8388	78.5234	30	147	15.267	4.765823	4.859637	4.81273
39	17.4934	104.826	78	135	17.498	4.719459	5.569786	5.144622

40	7.3729	101.3037	72	130	11.4113	3.063897	3.63233	3.348114
41	14.1036	104.0977	72	141	14.1659	4.237601	4.509146	4.373373
42	15.6115	103.2317	78	129	14.6936	4.458383	4.677118	4.567751
43	10.5391	104.307	70	136	12.0011	3.663168	3.820069	3.741618
44	9.1775	101.7648	67	139	11.6497	3.418356	3.708215	3.563285
45	10.4741	95.4692	63	125	13.1662	3.651854	4.190932	3.921393
46	9.6693	111.467	77	135	13.6705	3.508751	4.351455	3.930103
47	9.9904	107.7327	76	134	11.5427	3.566535	3.674156	3.620345
48	16.0464	95.7343	68	129	15.0022	4.520057	4.775349	4.647703
49	9.9904	112.9402	93	144	12.5931	3.566535	4.008508	3.787522
50	13.685	116.4758	94	140	13.4104	4.17424	4.268663	4.221452
51	16.9934	104.6826	77	140	15.3734	4.651523	4.893505	4.772514
52	12.4494	113.4851	91	133	14.2887	3.98134	4.548234	4.264787
53	7.4135	113.79	88	131	11.3794	3.072322	3.622176	3.347249
54	8.4947	111.9344	90	138	10.8884	3.288737	3.465885	3.377311
55	8.8402	96.2423	71	123	15.0734	3.35495	4.798012	4.076481
56	10.6854	104.9	74	132	11.8719	3.688506	3.778943	3.733724
57	14.1361	103.891	78	138	13.6136	4.242481	4.333343	4.287912
58	12.9737	99.49	75	135	13.3354	4.064311	4.24479	4.154551
59	13.7094	104.2656	70	144	14.7086	4.17796	4.681893	4.429926
60	9.5474	108.1652	68	134	11.2664	3.486564	3.586207	3.536385
61	13.1647	94.7326	63	127	14.0175	4.09412	4.461909	4.278014
62	18.0786	96.2394	66	133	15.9784	4.797748	5.086083	4.941915

Sample: 12.029.2.1

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	46.7451	78.3232	53	114	25.2988	7.714772	8.052858	7.883815
2	59.4425	78.6461	55	119	27.8798	8.699686	8.874416	8.787051
3	28.5446	73.7522	52	104	19.7455	6.028608	6.285188	6.156898
4	30.5199	74.3376	57	119	20.4706	6.23371	6.515994	6.374852
5	60.3001	76.1329	49	111	28.8598	8.762218	9.18636	8.974289
6	39.8722	74.2337	50	117	24.6073	7.125087	7.832747	7.478917
7	54.2156	82.0763	56	113	27.3591	8.308396	8.708672	8.508534
8	53.1954	78.5063	52	114	27.2195	8.229853	8.664236	8.447045
9	41.7256	84.1295	61	114	23.8535	7.288805	7.592805	7.440805
10	22.5414	81.1774	54	114	18.6789	5.357294	5.945679	5.651486
11	28.4999	75.924	53	113	19.3739	6.023886	6.166904	6.095395
12	15.1726	65.45	49	90	15.0703	4.395265	4.797025	4.596145
13	24.976	76.5177	56	113	18.6481	5.639187	5.935875	5.787531
14	30.6093	72.6375	50	110	20.1901	6.242834	6.426708	6.334771
15	40.6078	77.4868	51	121	23.5744	7.190512	7.503965	7.347238
16	25.4759	76.5841	58	105	18.2481	5.695342	5.808551	5.751946
17	41.3923	73.9509	49	107	24.3275	7.259636	7.743684	7.50166
18	30.2516	74.7435	48	108	20.0442	6.20625	6.380267	6.293258
19	33.43	58.4469	29	83	21.8833	6.52414	6.965671	6.744905
20	39.0227	73.7333	54	101	22.9222	7.048776	7.296363	7.172569
21	47.3142	80.9444	59	115	25.258	7.761592	8.039871	7.900732
22	39.7665	72.1856	55	109	23.1674	7.115636	7.374412	7.245024
23	37.0596	73.9891	49	106	22.3022	6.869188	7.099011	6.9841
24	31.6783	71.652	53	112	20.4261	6.350911	6.50183	6.42637
25	58.9872	75.6499	52	112	28.7277	8.666305	9.144311	8.905308
26	40.616	69.1199	42	115	23.4294	7.191238	7.45781	7.324524
27	56.955	79.2478	55	121	29.7103	8.515712	9.457082	8.986397
28	45.9851	73.3946	53	105	24.8605	7.6518	7.913343	7.782572
29	36.2955	77.8191	56	116	22.8054	6.798005	7.259184	7.028594

Sample: 12.029.4.1

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	10.4334	79.2855	48	118	11.8073	3.644752	3.75838	3.701566
2	9.8441	81.5764	56	120	11.9562	3.540324	3.805777	3.673051
3	12.5225	79.4619	53	110	13.2176	3.993012	4.207293	4.100152
4	10.1611	53.5528	38	77	13.9606	3.596876	4.443797	4.020336
5	3.9425	74.8763	55	127	7.6675	2.240479	2.440641	2.34056
6	6.5478	85.0441	62	111	9.7911	2.887372	3.116604	3.001988
7	6.4909	78.4652	48	122	9.5255	2.874799	3.032061	2.95343
8	11.1	82.9348	58	122	12.7516	3.759383	4.05896	3.909172
9	8.8036	78.4945	53	112	11.0539	3.347998	3.518566	3.433282
10	14.7336	78.3743	48	126	14.3505	4.331213	4.567906	4.449559
11	5.4301	79.1243	64	134	8.9201	2.629414	2.839356	2.734385
12	13.7216	72.7722	49	121	14.6613	4.179819	4.666837	4.423328
13	11.7218	72.4934	50	99	12.5996	3.863245	4.010577	3.936911
14	20.0905	73.2082	44	121	17.1936	5.057669	5.472893	5.265281
15	13.0834	72.972	45	105	13.8512	4.081458	4.408974	4.245216
16	14.2337	78.3946	54	118	13.637	4.257101	4.340792	4.298947
17	6.434	89.9128	64	124	9.6202	2.862171	3.062205	2.962188

Sample: 12.029.5.1

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	103.7327	104.9004	43	152	37.3447	11.49246	11.88719	11.68982
2	49.9926	114.1567	77	173	25.7792	7.978255	8.205774	8.092015
3	32.1579	109.6695	75	151	21.1737	6.398805	6.739798	6.569302
4	26.8863	109.8907	76	162	19.0831	5.850872	6.074339	5.962606
5	35.6492	111.9372	77	153	22.2538	6.737208	7.083605	6.910406
6	45.0096	120.8884	85	157	24.8177	7.570205	7.899719	7.734962
7	34.8973	114.9563	83	156	23.0573	6.66578	7.339367	7.002573
8	30.6378	108.1434	78	144	20.4371	6.245739	6.505331	6.375535
9	36.4255	109.293	69	155	22.5783	6.810168	7.186896	6.998532
10	28.3251	113.445	74	151	19.1741	6.005384	6.103306	6.054345
11	69.7458	117.2336	79	161	31.0221	9.42354	9.874641	9.649091
12	31.0849	112.6437	75	154	24.5035	6.291146	7.799706	7.045426
13	26.8944	110.1978	75	184	19.3325	5.851753	6.153726	6.002739
14	38.2301	110.297	77	163	22.3721	6.976824	7.121261	7.049042
15	46.7655	109.2769	70	155	27.7031	7.716455	8.818171	8.267313
16	62.5314	108.9654	74	151	28.8292	8.922861	9.176619	9.04974
17	97.7011	112.5485	79	161	36.0082	11.15334	11.46177	11.30755
18	37.3482	124.4405	74	162	23.2696	6.895883	7.406944	7.151413
19	46.1843	117.5217	78	168	24.8188	7.668356	7.900069	7.784212
20	24.8825	126.9992	82	170	19.0119	5.628622	6.051676	5.840149
21	32.2066	113.9107	80	148	21.3014	6.403649	6.780446	6.592047
22	30.8898	115.2355	74	162	20.9787	6.271373	6.677728	6.47455
23	26.6587	108.0666	76	153	19.8133	5.826055	6.306769	6.066412
24	21.1432	106.0233	77	149	17.1788	5.188483	5.468182	5.328333
25	29.52	107.2572	69	145	20.4294	6.130745	6.50288	6.316812
26	55.7154	111.9217	74	154	28.0235	8.422532	8.920157	8.671345
27	41.2785	120.9618	74	162	23.6054	7.24965	7.513832	7.381741
28	45.8754	115.1281	78	168	25.6215	7.642668	8.155577	7.899122
29	53.6384	106.2564	64	156	26.9935	8.264051	8.592298	8.428174
30	23.6347	114.5634	77	149	19.57	5.485675	6.229324	5.8575
31	54.5814	114.7954	75	156	26.6036	8.336378	8.468189	8.402283
32	52.9922	118.9933	81	159	28.8715	8.21412	9.190084	8.702102
33	24.346	111.7511	80	149	22.8393	5.567611	7.269975	6.418793
34	50.208	100.633	64	146	25.7225	7.995424	8.187726	8.091575
35	50.7486	121.6945	83	156	26.0028	8.038353	8.276948	8.157651
36	45.9038	119.2133	82	155	26.0874	7.645033	8.303877	7.974455
37	54.7318	111.2249	74	148	29.0044	8.347856	9.232387	8.790121
38	53.0775	104.1626	70	138	27.6626	8.220728	8.805279	8.513004
39	44.8105	110.979	72	151	25.6467	7.553443	8.163598	7.858521
40	48.2327	119.4264	81	160	27.2697	7.836567	8.680215	8.258391

41	39.5714	116.9815	75	150	26.2413	7.09816	8.352865	7.725512
42	27.5203	114.5497	78	157	19.8525	5.919454	6.319247	6.11935
43	33.6698	113.4965	74	160	21.7578	6.547497	6.925723	6.73661
44	53.988	119.3007	81	159	26.6017	8.290938	8.467584	8.379261
45	54.3741	115.429	55	166	28.5704	8.320532	9.094241	8.707386
46	36.08	118.5874	84	163	23.2204	6.777793	7.391283	7.084538
47	14.441	107.7166	71	145	15.1831	4.287989	4.832931	4.56046
48	39.9738	103.237	77	139	23.1234	7.134159	7.360407	7.247283
49	27.9877	100.3603	71	149	19.6019	5.96951	6.239479	6.104494

Sample: 12.029.5.2

	Area	Mean	Min	Max	Perim.	Diam (using A)	Diam (using P)	Average D
1	38.7504	118.1021	77	164	23.3487	7.02414	7.432122	7.228131
2	33.0236	115.3534	84	160	21.7263	6.484362	6.915696	6.700029
3	43.9285	115.4209	78	162	24.6024	7.478737	7.831187	7.654962
4	39.9819	113.4704	81	167	23.0577	7.134882	7.339494	7.237188
5	36.2548	111.8413	76	187	22.0251	6.794192	7.010807	6.9025
6	45.1885	115.2806	77	161	25.1942	7.585235	8.019563	7.802399
7	37.3034	108.719	75	147	22.3618	6.891746	7.117982	7.004864
8	22.0496	113.9935	76	146	17.6804	5.29853	5.627846	5.463188
9	29.2071	114.6618	75	161	21.8433	6.098167	6.952938	6.525552
10	34.0031	114.6749	77	184	23.7404	6.579825	7.556804	7.068314
11	39.4454	115.3813	70	156	23.2255	7.08685	7.392906	7.239878
12	32.1822	110.4684	79	171	21.1279	6.401223	6.725219	6.563221
13	31.8734	113.1368	71	174	20.7674	6.370437	6.610469	6.490453
14	43.9488	120.2915	75	166	24.5195	7.480465	7.804799	7.642632
15	29.6542	111.9729	74	154	19.633	6.144664	6.249378	6.197021
16	34.0844	119.9174	87	152	21.6066	6.587686	6.877594	6.73264
17	30.6825	119.3171	79	173	20.3406	6.250294	6.474614	6.362454
18	97.0142	120.4398	75	171	37.0482	11.11406	11.79281	11.45343
19	31.8083	115.5985	86	164	21.6158	6.363928	6.880523	6.622226
20	29.5282	105.9597	74	166	20.0822	6.131596	6.392363	6.261979
21	41.819	121.4377	77	169	24.1327	7.296959	7.681677	7.489318
22	39.2625	115.3763	79	149	23.3813	7.070401	7.442499	7.25645
23	27.8292	118.8329	75	168	19.1679	5.952582	6.101332	6.026957
24	29.0486	107.9723	70	156	19.9438	6.081597	6.348309	6.214953
25	27.0895	111.6272	77	156	20.0647	5.87294	6.386792	6.129866
26	32.796	106.4655	78	144	20.926	6.461978	6.660953	6.561466
27	70.0466	110.6104	74	165	31.8554	9.443839	10.13989	9.791864
28	57.0688	114.0022	74	166	28.1336	8.524216	8.955203	8.739709
29	42.5059	126.0273	70	178	23.4762	7.356643	7.472707	7.414675
30	34.2389	113.1668	75	154	22.2183	6.6026	7.072305	6.837452
31	65.3115	117.6714	75	187	30.478	9.119056	9.701449	9.410252
32	33.2106	111.9217	77	156	21.3638	6.502696	6.800309	6.651502
33	30.4752	93.6727	55	137	20.0183	6.229144	6.372023	6.300583
34	43.9935	120.361	78	173	24.196	7.484268	7.701826	7.593047
35	26.7156	124.6413	79	156	19.2972	5.832269	6.14249	5.987379
36	29.1624	117.1236	83	155	19.7754	6.093498	6.294705	6.194102
37	47.7613	111.0201	74	167	26.4454	7.798178	8.417832	8.108005
38	33.2675	125.3146	78	173	22.6745	6.508264	7.217518	6.862891
39	59.5766	122.0439	80	162	28.9512	8.709494	9.215453	8.962474
40	40.1282	118.9754	73	158	23.8587	7.147924	7.59446	7.371192

41	68.7622	114.5843	74	164	30.4954	9.356856	9.706987	9.531922
42	45.916	113.2266	70	151	25.104	7.646049	7.990851	7.81845
43	40.0226	112.3921	80	151	24.4092	7.138512	7.76969	7.454101
44	31.4466	116.6565	76	165	20.9581	6.327642	6.67117	6.499406
45	20.054	118.3468	81	158	16.5035	5.053073	5.253227	5.15315
46	38.161	120.8741	82	156	22.4418	6.970516	7.143447	7.056981
47	48.5741	121.8033	78	173	25.0368	7.864252	7.969461	7.916857
48	33.6007	115.9354	79	163	21.8432	6.540775	6.952907	6.746841
49	52.9272	122.872	80	162	28.3266	8.209081	9.016637	8.612859

APPENDIX D – HISTOLOGY PROTOCOL

Block Information

####: _____

Materials

Section

- ____ 1. Forceps (2)
 ____ 2. Brushes (2)
 ____ 3. Water bath (1)
 ____ 4. Microtome (1)
 ____ 5. Blade (1)
 ____ 6. Cassette (1)
 ____ 7. Slides (?)

Staining

- ___ 8. xylene (3)
- ___ 9. 95% (2)
- ___ 10. 100% (2)
- ___ 11. H₂O (2)
- ___ 12. Hematoxylin (1)
- ___ 13. Bluing (1)
- ___ 14. Clarifier (1)
- ___ 15. Eosin (1)
- ___ 16. Slide basket (1)
- ___ 17. Cover slips (?)
- ___ 18. Hemostats (1)
- ___ 19. Glue

Description-Section

Prepare tissue for later analysis. Turn on water bath, set-up microtome, and position blade and cassette. Get sections on glass slips, label, and let air dry.

Protocol-Staining

- ____ 20. Heat slides in oven till clear
 ____ 21. 3 min xylene 3x
 ____ 22. 2 min 100% 2x
 ____ 23. 2 min 95%
 ____ 24. 1 min H₂O
 ____ 25. 4 min Hematoxylin
 ____ 26. 1 min H₂O

- ____ 27.30 sec Clarifier
 ____ 28.1 min H₂O
 ____ 29.1 min Bluing
 ____ 30.1 min H₂O
 ____ 31.1 min 95%
 ____ 32.1 min 30 sec Eosin
 ____ 33.1 min 100% 3x
 ____ 34.3 min xylene 3x

Diagram

xylene	95%	H₂O	95%
xylene	100%	Hematoxylin	Bluing
xylene	100%	H₂O	Clarifier
	Eosin		

Notes

[illegible]