THE IMPACT OF INCREASED COLLATERAL FLOW ON TRANSVERSE
ARTERIOLAR MICROVASCULAR TREE MORPHOLOGY

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By

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TITLE: THE IMPACT OF INCREASED COLLATERAL FLOW
ON TRANSVERSE ARTERIOLAR
MICROVASCULAR TREE MORPHOLOGY

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Abstract

The Impact of Increased Collateral Flow on Transverse Arteriolar Microvascular Tree Morphology

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The goal of this study was to understand how arteriogenesis impacts transverse arteriolar tree structure. The previous process involved a vascular casting technique that utilized black India ink as the casting agent and manual measurement of vessel morphology with ImageJ. This study decreased the complexity of the casted samples by using a casting compound called Microfil. Microfil's viscosity can be adjusted and through experimentation, a viscosity was found that stopped at the capillary level, allowing only the arterial side of the circulation to be casted. A macro in ImageJ was written to help with morphological measurement by bundling vessel length and diameter into one measurement instead of two. These two adjustments were then used in a ligated mouse model to compare healthy and remodeled vascular networks. The analysis revealed that the only significant difference for length and diameter measurements was in the diameters of the collateral. This indicates that it's possible that stimulating arteriogenesis in a clinical environment should not cause any negative effects in the collateral-containing muscle. However, this observation is potentially limited by the differences between the model and patient.

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“A master in the art of living draws no sharp distinction between his work and his play; his labor and his leisure; his mind and his body; his education and his recreation. He hardly knows which is which. He simply pursues his vision of excellence through whatever he is doing, and leaves others to determine whether he is working or playing. To himself, he always appears to be doing both.”

~Lawrence Pearsall Jacks

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Chapter 1 - Introduction

Normal Function of Circulatory System

Overview

The primary function of the circulation is to carry out nutrient and waste exchange in all of the body tissues, and the rate of blood flow is controlled by tissue demand for oxygen and nutrients. Additional functions include the transport of endocrine factors, thermoregulation, and inflammation (17, 24, 32). The blood flow and circulation in the body are divided into the systemic circulation and the pulmonary circulation, the systemic circulation is also known as the peripheral circulation (**Figure 1**) (17).

Figure 1 – Basic circulatory patterns of blood flow. Adapted from (9).

The pump that creates the circulatory flow is the heart. The human heart is made up of 4 major chambers: the right atrium, right ventricle, left atrium and left ventricle (17, 32). The heart also has 4 valves; each valve is unidirectional and keeps blood in the heart flowing in the one direction. The valves are made up of two or three small but strong flaps of connective tissue called leaflets. The leaflets open to allow blood to flow through the valve and close to prevent blood from flowing backwards (17, 32), depending on blood pressure gradients across their location. The tricuspid valve is positioned between the right atrium and the right ventricle. The pulmonic valve separates the right ventricle from the pulmonary artery. The mitral valve is positioned between the left atrium and the left ventricle and the aortic valve separates the left ventricle from the aorta (Figure 2).

Figure 2: Human Heart Diagram, adapted from (21)

The process of blood circulation begins with the right side of the heart receiving blood that is low in oxygen from the vena cava, which drains veins from all over the body. It then pumps the blood through the pulmonary arteries into the lungs where it will become oxygenated. The oxygenated blood then leaves the heart from the aorta and travels through a branching system of arteries and arterioles; from the arterioles the blood travels into capillaries, the site of nutrient and waste exchange, before moving into venules; the venules gradually coalesce into veins, which bring the deoxygenated blood back to the vena cava and right atrium to form a continuous circulation (3, 12, 17). At each order of branching, the sizes of the vessels decrease but the number of individual vascular entities increases (12, 17) and all blood in the human body will traverse the full circuit an average of once each minute when the body is at rest and as many as six times each minute during physically activity (17).

The role of the arteries is to transport blood under high pressure and velocity to the tissues, compared to the veins, which transport blood at high velocities but low pressures and thus form the capacitance vessels of the circulation (as much as 60% of a person's blood volume can be found in the veins at rest). The arterioles are the last of the small branches of the arterial system before blood flows into the capillaries (32), the primary site of nutrient and waste exchange between the blood and the interstitium/parenchyma. The venules then collect the blood from the capillaries as they gradually coalesce into veins. Veins take the deoxygenated blood back to the heart to be oxygenated by the lungs and also serve as a major reservoir of extra blood (17).

Microcirculation

As stated earlier, the most important functions of the circulation occur in the microcirculation. This is due to the fact that the microcirculation is the site of nutrient and waste exchange. Capillary blood flow is determined at the upstream arterioles, based on the contractile state of smooth muscle cells that encircle the arterioles. Smooth muscle cells control arteriolar diameter by either contracting to constrict arterioles and decrease blood flow, or relaxing to dilate the arterioles and increase blood flow. Arterioles have the potential to drastically changing the blood flow in response to tissue needs (17) because according to Poiseuille's Law, diameter is the largest contributing factor to blood flow: $F = \frac{\pi \Delta P r^4}{8 \eta l}$, where F is the rate of blood flow, ΔP is the pressure difference between the ends of the vessel, r is the radius of the vessel, η is the viscosity of the blood, and l is the length of the vessel. It is important to note that blood flow is proportional to the fourth power of the radius of the vessel, which is why it is the largest factor when determining blood flow for the tissue for which it is supplying blood (17). The arterioles will branch until they reach diameters of 5 to 9 micrometers where they transition to capillaries (32). The walls of the capillaries are extremely thin, constructed of single-layer, unicellular endothelial cells, which are surrounded by a very thin basement membrane on the outside of the capillary (32). Although it isn't necessarily continuous like arteriolar smooth muscle, capillaries also have perivascular cells that surround the endothelium, but do not contain smooth muscle contractile proteins like arterioles (17, 32). The total thickness of the capillary wall is only about 0.5 μm . The internal diameter of a capillary is 4 to 9

micrometers, barely large enough for red blood cells and white blood cells to squeeze through (17, 32). Therefore, water, cell nutrients, and cell waste can all interchange quickly and easily between the tissues and the circulating blood. Diffusion is the most common way that substances are transferred between the interstitial fluid. If a substance is lipid soluble, like oxygen and carbon dioxide, in specialized microcirculations, such as the liver and kidney, it can diffuse directly through the cell membranes of the capillary without having to go through the pores. This process is much faster than for substances like sodium ions and glucose that are lipid-insoluble and must diffuse through the inter-cellular junctions or endothelial transport proteins (10, 17, 35). The human body has about 10 billion capillaries with a total surface area estimated to be around 500 to 700 m² with a total cross-sectional area of 2500 cm², while the total cross-sectional area of the aorta is only 2.5 cm² (3, 17, 24). This is why branching plays such a crucial role in efficient blood delivery; it allows the body to effectively deliver crucial nutrients and expel harmful waste over a large area from a small initial cross-sectional area.

Dysfunction in the Circulatory System

Peripheral Artery Disease

Ischemic vascular diseases are the most common cause of death in the western world (19). The vascular endothelium is sensitive to changes in physical forces (e.g. shear) or signaling molecules, both of which can result in pathology (24).

Peripheral arterial disease (PAD) is an obstructive arterial disease that causes ischemia in the lower extremities during exercise or, in more severe cases, during rest (46, 50). PAD is a highly prevalent atherosclerotic disease, most common in aged populations (20, 46, 50), affecting 8 to 12 million Americans. PAD is associated with significant morbidity and mortality (20, 50), mostly due to a significant increase in coronary artery disease in these patients. PAD can be categorized as either intermittent claudication, which involves pain during physical activity, or critical limb ischemia, which involves a severe blockage in the arteries of the lower extremities that results in pain at rest (20, 46, 50). Complications can include chronic ulcerations in the legs and feet and if left untreated, the complications of critical limb ischemia will result in amputation of the affected limb (**Figure 3**).

Figure 3 – Chronic limb ischemia. Adapted from (22).

The pathogenesis of PAD is due to atherosclerosis (17, 20, 32, 37, 46, 50). Atherosclerosis is a disease of the medium and larger sized arteries in which fatty lesions called atheromatous plaques develop on the endothelial surfaces of the arterial wall (17, 32). Endothelial dysfunction is a precursor to atherosclerosis and can be measured before plaques form. Endothelial dysfunction involves the increased expression of adhesion molecules and a decrease in nitric oxide and prostacyclin that normally prevent adhesion of platelets and monocytes (2, 17, 41). Circulating monocytes and lipids then aggregate at the site of the intimal injury; eventually the monocytes extravasate and differentiate into macrophages that phagocytose oxidized lipoproteins that have accumulated in the subintimal space (2, 41). Ingestion of the lipoproteins by macrophages will lead to a fatty streak and with time the fatty streak will grow larger and larger, which will cause the surrounding smooth muscle and fibrous tissues to proliferate to form plaques (2, 17). In addition, macrophages also release inflammatory cytokines that stimulate smooth muscle cell proliferation and expansion of fibrous tissue in the subintimal space (2, 41). The cellular proliferation coupled with the lipid deposits can become so large that the plaque bulges into the lumen of the artery and reduces blood flow; severe cases will completely occlude the vessel (17). Normally, arteries are very elastic, but eventually atherosclerosis causes arteries to become stiff, referred to as “hardening of the arteries,” due to connective tissue accumulation and calcium salts precipitating with cholesterol and other lipids causing hard calcifications (17). Once the arteries have hardened, the plaques they contain are at risk for rupture; their rough surface also protrudes

into the flowing blood, causing blood clots to develop that block the flow in the artery (17, 32, 41). Patients with PAD have a much higher risk of coronary heart disease; therefore the treatments aim to decrease cardiovascular risk while also restoring the patient's quality of life by reestablishing blood flow to the ischemic limb (20, 46).

Patient Treatment

Most of the treatments for PAD are designed as secondary prevention for cardiovascular risk. These treatments include: supervised exercise, smoking cessation treatments for patients that are smokers, antiplatelet therapies, blood glucose control for patients with diabetes, and lipid-lowering agents (20, 37, 46). Other more serious treatments that address primary risk include percutaneous coronary re-vascularizing interventions, coronary bypass surgeries, endarterectomies, peripheral stenting and by-passing. In 1999, in the US alone, there were 1.1million percutaneous coronary revascularizations, 355,000 coronary bypass surgeries and 131,000 endarterectomies (19). These serious treatments have their limitations and risks, for example, not all patients with coronary and peripheral vascular disease are able to receive these therapies due to age or unavailability of a vessel for a bypass. Also, about one third of patients will develop restenosis after treatment, and the neurological risk for patients that need to be placed on cardiopulmonary bypass for their surgery is high (19). While all of these treatments are somewhat successful they fail to stimulate the

vasculature's natural repair process of arteriogenesis and angiogenesis to restore blood flow to the ischemic limb.

Therapeutic angiogenesis and therapeutic arteriogenesis were proposed as treatment strategies in the early 1990s as an alternative to surgical or endovascular revascularization therapies. The goal of therapeutic angiogenesis/arteriogenesis is to treat ischemic disease by stimulating the natural revascularization processes of the body. This would cause a restoration of blood flow to an occluded tissue, which is an important but challenging goal as there are several hemodynamic, metabolic as well as cellular and molecular signaling factors involved in the mechanisms of structural remodeling and adaptation in the microcirculation (24, 29, 30). The ultimate goal is to understand the mechanisms of vascular growth in order to develop treatments to stimulate arteriogenesis and angiogenesis as a single therapy or even as an addition to other surgical procedures to achieve a more complete level of revascularization for both macrovascular and microvascular diseases.

Vascular Growth

There are many different aspects to vascular growth, which can be separated into three categories: vasculogenesis, angiogenesis and arteriogenesis. Each of these aspects can then be broken down further into different entities with different mechanisms, which will be discussed later. Understanding each of these aspects is crucial for developing tests and testing strategies that help create efficacious treatments.

Angiogenesis

Angiogenesis is defined as the growth and development of new capillaries from existing capillaries and is a normal process critical to development, wound healing, reproduction, and exercise adaptation (3, 12, 15, 19, 24, 38, 51). It is also involved in inflammatory diseases such as rheumatoid arthritis, psoriasis, and tumor growth (19). Angiogenesis is categorized by two different types of vessel growth: sprouting angiogenesis and intussusceptive angiogenesis (12, 40). In the embryo, the vascular system begins with vasculogenesis, the de novo creation of capillaries by endothelial and perivascular cells. Vasculogenesis (**Figure 4A**) is followed by a sprouting phase (**Figure 4B**) characterized by the appearance of multiple capillary sprouts that then fuse to form the primary capillary plexus (**Figure 4C**) that is remodeled into an optimal circulation by intussusception (12, 13, 24, 40). In the adult, sprouting angiogenesis occurs primarily in response to hypoxia while intussusception is stimulated by increased shear stress (12, 13, 18, 40).

Intussusceptive angiogenesis involves the formation of new capillaries by the development of tissue pillars in the capillary lumen and expansion of those lumens to form new capillary networks (12, 38, 51). Further, both vascular growth and remodeling occur mostly by intussusception (12, 13). The distinguishing factor that separates sprouting angiogenesis and intussusceptive angiogenesis is the formation of a tissue pillar into the vascular lumen. There are four consecutive steps in pillar formation: first, a zone contact is established between opposite capillary walls; second, there is a reorganization of the inter-endothelial

cell junctions and central perforation of the bilayer; the third step is the formation of the interstitial pillar core; and the final step is the increase in girth of the pillar (3, 12). Intussusceptive angiogenesis is pervasive and can occur in many organs during normal (cerebral vascularization after stroke) and pathological (tumor angiogenesis) microvascular growth in order to establish normal branching patterns (12, 38).

Once the pillar formation has occurred there are three distinct elements that define intussusceptive angiogenesis. The first is intussusceptive microvascular growth, which can be described as the expansion of the capillary plexus (12). The next element is intussusceptive arborization, which is the formation of a vascular feeding tree. The original capillary plexus formed by vasculogenesis and sprouting doesn't resemble a tree-like branching pattern, where larger arteries branch into progressively smaller and smaller diameter conduits, which is the most efficient flow delivery scheme. Intussusceptive arborization is the process by which the capillary plexus is transformed into a tree-like branching pattern after intussusceptive microvascular growth has taken place. Intussusceptive arborization is initiated by the formation of compacted vertical pillars; these pillars undergo reshaping into narrow tissue septa that progressively fuse to delineate a new vascular entity (12). The final element is intussusceptive branching remodeling, which is how the vascular tree geometry is optimized (13). The function of intussusceptive branching remodeling is to optimize hierarchy and flow properties within the system by minimizing power consumption and maintaining a constant shear stress, this occurs by changes in

branch angle through the formation of transluminal pillars near bifurcation sites of arteries and veins (13). Another aspect of intussusceptive branch remodeling is the removal of branches that are unnecessary by a mechanism called vascular pruning, or regression (38), where large numbers of smaller vessels are reduced for few larger sized ones (**Figure 4D**) (4, 12, 13). The cause of pruning can be attributed to hyperoxia, which causes VEGF to be down-regulated and unnecessary vessels to be pruned (38).

Figure 4 – Diagram of optimal circulation through angiogenesis. A) Vasculogenesis is the creation of capillaries by endothelial and perivascular cells. B) Sprouting marks the appearance of multiple capillary sprouts that then fuse to form the C) primary capillary plexus. Intussusceptive angiogenesis then begins with intussusceptive microvascular growth (IMG), or the rapid expansion of the capillary plexus. Next, intussusceptive arborization (IAR) forms the vascular feeding tree. Finally, intussusceptive branching remodeling (IBR) optimizes branching geometry through branch angle adjustments and D) pruning.

The effectiveness of pillar formation is dependent on blood flow rate, and the faster the blood flow rate is, the faster the pillar will form, and it is a process that occurs with minimal amounts of endothelial cell proliferation, while also maintaining low vascular permeability. In contrast, sprouting angiogenesis is a drawn out process that requires extensive proliferation of endothelial cells and an increased vascular permeability , which expends more energy (12).

Arteriogenesis

Angiogenesis is the formation of new capillaries by sprouting and intussusception from pre-existing architecture, and vasculogenesis is the embryonic development of blood vessels from angioblasts (4). Arteriogenesis, on the other hand, is a different process by which a small arteriole chronically increases its diameter through outward remodeling (4, 38). Angiogenesis and arteriogenesis have different initial pathways as well as different results; the main difference is that arteriogenesis is more effective than angiogenesis in replacing an occluded artery by returning normal blood flow to an ischemic tissue (4). The outward remodeling occurs through endothelial cell activation, vascular cell proliferation, and rearrangement of the extracellular matrix (38).

Increased sheer stress is a trigger for increases in arterial diameter (flow induced dilation), and with the change in flow that persists, outward remodeling of the arterial wall will occur to normalize hemodynamic shear stress (4, 19, 38). It makes sense then, that the maximal arteriogenesis occurs in situations at maximal sheer stress (4). The increased flow increases the arterial wall tension

and activates the endothelium (4). What is still unclear is how the increase of shear stress is being “sensed” by the cells of the vascular wall (4, 19). The endothelium play a crucial role in the remodeling response to shear stress as the first sign of arteriogenesis after arterial occlusion is that the endothelial cells of collateral vessels appear swollen and are randomly oriented and do not line up in the direction of blood flow (4). Endothelial cells are able to react to different shear stress gradients by activating multiple transcription factors (19). Once the endothelium is activated, there is an increase in endothelial nitric oxide (NO) synthase, monocyte chemoattractant protein (MCP-1), transforming growth factor beta (TGF- β), as well as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM) (4, 19, 43). The up-regulation of MCP-1 seems to be important for the attraction of monocytes to the endothelium and their aggregation around growing collateral vessels (4, 19). The effects of NO are not yet understood, but it has been found to dilate collateral vessels, which decreases shear stress. NO can also activate metalloproteinases (MMPs) and may play a role in cell migration and proliferation, on the other hand, NO can also have inhibitory effects on endothelial, vascular smooth muscle cells (VSMC), atherogenesis, and inflammation (19). There is also an increased permeability of the endothelium, as can be seen from the adherence of monocytes, and leakage of platelets, plasma proteins and erythrocytes (4, 42). Other molecules that have been found to be involved are MMP-2, FAK, and bone marrow tyrosine kinase (4), which are involved in cell proliferation and migration (4).

Increased shear stress also changes VSMC during arteriogenesis by converting smooth muscle cells (SMC) between contractile (physiological phenotype) and synthetic (proliferative phenotype) (4, 47). The cellular signals to mobilize the SMC come from the degradation of the basement membrane, and the internal and external elastic laminae, which act as barriers to prevent outward remodeling of the vessel. In order for this degradation to occur MMP-2 and MMP-9 are up-regulated in the SMC, which causes the basement membrane and the internal and external elastic laminae to become fragmented to allow for collateral vessel growth (4). Other signals come from transcriptional regulators like ERK and FAK, which promote cellular migration (4). The mechanical strain from the occlusion, increased shear stress and growth factor stimulation lead to the autophosphorylation of FAK and ERK to promote the migration and proliferation of VSMC so that arterioles' successfully transform into collateral vessels (**Figure 5**) (4).

Figure 5 – The process of arteriogenesis. Adapted from (38).

It is important to appreciate that arteriogenesis is more effective than angiogenesis in replacing an occluded artery by returning normal blood flow to an ischemic tissue (4) and understanding the entire process is crucial to advancing the field of therapeutic arteriogenesis for diseases like PAD.

Conclusion of Vascular Growth

All of the remodeling processes are a result of complex and coordinated cellular interactions that are brought about by cell-to-cell and cell-to-matrix communication from environmental stimuli, so that constant blood vessel remodeling occurs (38), where network structure is remodeled to match tissue requirements . Understanding how vascular growth works and how different therapies affect the long-term stability and survival of tissue in patients with ischemic diseases is critical to designing both revascularization therapies for ischemic disease (and anti-angiogenic therapies for cancer) (38, 51). As we progress in therapeutic arteriogenesis and angiogenesis there are important considerations that need to be made: delivery methods and agents need to be effective in stimulating arteriogenesis but also have acceptable levels of risk, e.g. plaque instability, plaque growth, or an inflammatory response. We must weigh success from our vascular growth treatments with treatment failure from tumor growth or other inflammatory diseases, and decide how much of that risk is acceptable when searching for success (19). As we move forward and continue to gain insight into vascular growth and the role that it plays in other diseases, there is the potential that this type of research could change the face of medicine

in the next decades, with more than 500 million people worldwide predicted to benefit from pro or anti-angiogenesis treatments (15).

Attempts to Stimulate and Assess Vascular Growth

It is important in the development of treatments that we are able to both stimulate and assess vascular growth. With this goal come many challenges as we make progress toward developing vessel maturation therapies for therapeutic angiogenesis. One hope for patients who are suffering with various ischemic diseases is the ability to form vascular networks *in vivo* by delivering angiogenic or arteriogenic molecules as recombinant proteins using controlled-release devices or as genes using viral vectors or plasmid DNA (23). Results from clinical trials using these techniques have shown that the formation of a mature vascular network will require very precise regulation of a large number of both angiogenic stimulators and inhibitors (24). For example, clinical trials for limb ischemia using VEGF and FGF yielded less than optimal results, as some patients had successful limb salvage while a majority of the patients treated with VEGF developed transient limb edema, which is consistent with VEGF enhancement of vascular permeability (1). Preclinical experiments using a controlled release device or an adenoviral vector to deliver basic FGF or VEGF led to the transient development of stable vascular networks, which after time began to resemble an immature tumor vasculature (11, 36). Therefore, more precise understanding of the vascular growth process through the use of animal models is necessary before therapeutic angiogenesis will be successful.

Many follow up experiments have focused on a more precise approach as the lack of positive trial data has focused researchers and clinicians on the fact that arteriogenesis and angiogenesis are complex processes and simply supplying vascular growth factors to ischemic tissue does not provide effective therapy (49).

One approach to improve the effectiveness of therapeutic angiogenesis is to deliver a cocktail of angiogenic molecules to the desired site in a spatiotemporal manner that more accurately resembles their endogenous production (24). Polymeric delivery systems can mimic the endogenous release of two or more growth factors. For example, controlling the dose and rate of delivery for a VEGF and platelet-derived growth factor (PDGF) combination therapy leads to vessels that appear more mature than those formed using VEGF alone (39). These results moved researchers to find out the minimal number and type of molecules needed to produce stable vascular expansion and to figure out how to optimize delivery in order to effectively treat ischemic disease. Another approach is to deliver a transcription or growth factor that regulates expression of the various growth factors required for mature vessel formation. Hypoxia-inducible factor (HIF)-1 α is responsible for regulating the expression of a large number of pro- and antiangiogenic genes and it enhanced perfusion in an ischemic rabbit hind-limb model (45, 48).

To be truly effective, once mature vessels have been established, therapeutic angiogenesis must successfully create a mature network structure. A mature network involves network remodeling and vascular reactivity. Network

remodeling involves arborization and pruning to create an efficient network (12, 31), while vascular reactivity is important for regulating blood flow in response to tissue needs (26). The ultimate goal of therapeutic angiogenesis is to revascularize ischemic tissue, which requires a mature vasculature and a mature network that will restore tissue oxygenation levels in response to the chronically insufficient blood supply.

The therapies described above are moving towards creating more mature vessels but little work is being done to create more mature networks, which is due to the difficulty of assessing a network. Most often, network maturation is assessed by counting vessels in a histological cross section, which gives little information on what the overall network looks like. In order to create more effective therapies it's important to understand how to create more mature networks.

Several morphological parameters such as the diameters of blood vessels, blood path length, vessel length, branching characteristics, and vessel quantity within a vascular network describe the architecture of the microcirculation. Following ischemia, these morphological parameters change. Diameters increase in response to the elevated shear stress, blood path length increases as blood is re-routed, which consequently causes a change in the branching characteristics of the network because of the structural remodeling and changes in flow direction. All of these changes could be important when developing therapies; however, researchers in this area typically ignore network topology. Instead of focusing on one morphological parameter, such as vessel number, it

would be beneficial to examine as many as possible in order to evaluate the vascular network as a whole. The microcirculation is critical to nutrient and waste exchange and changes could affect muscle functionality; the overall goal of this research was the changes in morphological parameters following arterial occlusion.

Objectives/Hypothesis

Currently, our lab uses a manual process to gather morphological data for our experiments. The morphological data is used in a model to predict hemodynamics and calculate vascular morphometry in normal and remodeled microcirculatory networks. This study aims to establish a system that will expedite the process of gathering morphological data needed for the model and to examine the impact of arterial occlusion on morphology. More specifically, this study had three distinct objectives to reach the goal:

- (1) Create a vascular casting protocol to only cast the arterial side of the microcirculation. Previous work filled both the arterial and venous sides, even though only the arterial side was measured. The venous fill increased the difficulty of measurement and tracing and provided no additional value to the research.
- (2) Decrease the amount of time it takes to measure the microvasculature of the anterior gracilis muscle through some form of automation. Previous work used completely manual measurements to gather data and I sought to expedite this data-gathering process.

(3) Determine the effects of elevated flow in a collateral artery on branching arteriolar network topology. This aim utilizes the proposed methods to demonstrate proof-of-concept and to determine if collateral remodeling was associated with remodeling in the arteriolar side branch trees.

Chapter 2 – Development of an Arterial-Specific Vascular Casting

Procedure

Introduction

Efficient morphological assessment requires a multi-step tissue preparation process in order to collect data for analysis and for input into our model. The process of sample preparation can be summarized in 5 steps: vascular casting, dissection, dehydration, clearance, and sample mounting. Improving the vascular casting protocol could optimize the entire process of gathering data for the model. The goal of vascular casting is to fill the circulation with a high contrast agent, which generally requires clearing the circulation of blood. The original protocol for vascular casting utilized nontoxic India ink(16). Although this protocol is successful in achieving high contrast between the vasculature and surrounding tissue, there are some drawbacks to casting with India ink and the subsequent morphological analysis, which include: India ink remains in a liquid state, so leakage occurs if the vessels aren't cauterized properly during muscle dissection; India ink takes a significant amount of time to dialyze, filter, and purify in preparation for injection; and India ink casts both the arterial and venous side of the circulation. I aimed to resolve these three issues to improve data collection. The solutions formulated for visualizing only the arterial side were to increase the viscosity of the casting agent to prevent it from traversing the capillary level by adding dextran to the casting compound or to use a fluorescent marker to label the arterial side of the circulation, so that when imaged the arterial side is expressed with fluorescence. While researching

different casting agents, I found a group that was able to successfully cast the spinotrapezius vasculature of a rat using Microfil (27). Further research into Microfil demonstrated that it was a radiopaque, polymerizing silicone rubber compound with variable viscosities that could be used to cast the arterial side of both a rat and mouse kidney (33). This previous work was successful using Microfil to cast the arterial side and led me to begin protocol modification with Microfil instead of modifying the India ink or using fluorescence.

Materials and Methods

Microfil is a casting agent with variable viscosity, composed of a colored compound, diluent, and curing agent. The optimal Microfil viscosity for arterial casting of the gracilis muscle was investigated using a murine model. All protocols were approved by the Institutional Animal Care and use Committee. Male Swiss Webster mice weighing 25-35g from the California Polytechnic State University Vivarium were used to optimize the Microfil casting protocol, 8 male C57BL/6 mice were used to validate the consistency of the protocol, and another 8 male C57BL/6 mice were used to assess the impact of arterial ligation on microvascular morphology (presented in Chapter 4).

The longitudinal gracilis arcade is the main blood supply for the gracilis muscle, and is fed by the profunda femoris of the femoral artery (medial side) and the saphenous artery (lateral side) (16). The gracilis arcade runs parallel to the muscle fibers over almost the entire length of the muscle (16) (**Figure 6**).

Figure 6 – The gracilis artery runs through the gracilis anterior and is fed by the profunda femoris and the saphenous artery.

Completing a vascular cast with Microfil requires the preparation of surgical instruments and materials. These included forceps, bone-cutting scissors, iris scissors, hemostats, microdissection scissors, a vascular clamp, surgical tape, 20ml syringe, bench cover, heating pad, catheter/stopcock/blunt needle apparatus containing a PE-100 catheter, isoflurane anesthetic, and gauze sponges (**Figure 7**). A vasodilator cocktail is also necessary to flush out the blood and maximally dilate the vasculature before injecting the casting agent.

Figure 7 – Microfil casting procedure set up. A) Light microscope, which can be rotated in to check on the progress of the vascular cast. B) Surgical instruments – Hemostats (1), Bone scissors (1), metzenbaum scissors (1), Iris scissors (1), forceps (2), microdissection scissors (1), vascular clamp (1). C) Syringe pump. D) Bench Cover with heating pad underneath. E) Octopus light source. F) Isoflurane nose cone. G) PE-100 catheter. H) Miscellaneous items-Saline, gauze, tape.

The cocktail consists of heparin, adenosine and sodium nitroprusside, which are all combined with PBS solution and heated to 37°C in a water bath. The Microfil mixture is created by vortexing 2mL MV-122, 1.25mL HV-Diluent and 1.25mL MV-Diluent into a 14mL conical and placing in the 37°C water bath (**Figure 8A**). Once the prep work was done the animal was weighed and anesthetized with isoflurane and laid supine upon a heating pad to maintain core body temperature. Hindlimb hair was removed with clippers and depilatory cream (**Figure 8B**). The

animal was taped down to the heated bench cover and the skin was separated from the superficial abdominal and thoracic musculature. Once the skin was removed, the 20mL syringe was filled with warm vasodilator cocktail, loaded into the syringe pump, and attached to the catheter. An incision was made through the abdomen, close to the diaphragm before the ribs and diaphragm were quickly transected and reflected with hemostats to expose the chest cavity (**Figure 8E**). Due to pneumothorax, the ventricular catheterization must be done quickly before blood begins clotting, which would ruin the clearance and vascular cast. Excess tissue was cut away from around the heart and a small incision was made in the apex of the left ventricle. The catheter was inserted through the incision into the left ventricle and clamped in place with a vascular clamp (**Figure 8C**). Once the catheter was in place the right atrium was cut and the syringe pump was started in order to inject the vasodilator cocktail at $5\text{mL}\cdot\text{min}^{-1}$. The skin above the gracilis muscle was removed to visualize vascular clearance before refilling the 20mL syringe to continue flushing the vasculature (**Figure 8G**). Once the entire vasodilator cocktail had been injected, .2mL of curing agent was added to the Microfil mixture to start the polymerizing reaction (it is crucial to wait until the last possible moment to add the curing agent when preparing the Microfil mixture to maintain the compound in a liquid state during perfusion). The Microfil mixture was inserted into the syringe pump and perfused at .5mL per minute (**Figure 8D**). The hindlimb was monitored under the microscope until the Microfil had passed the knee. At this point the syringe pump was stopped (approximately 1mL of Microfil had been perfused) and a tourniquet was tied around both ankles

with silk suture to prevent entry of the Microfil in the venous circulation through A-V shunts near the ankle (**Figure 8F**). The Microfil perfusion was resumed, and continued until the casting agent spilled out of the right atrium or until the cast was deemed satisfactory by visual inspecting the gracilis under the microscope. The catheter was removed, but the heart clamp was left to prevent any Microfil leakage, and the mouse was allowed to sit undisturbed for at least 10 minutes while the Microfil begin curing (**Figure 8H**). The open wounds were covered with plastic wrap and the mouse was placed in a plastic bag to prevent desiccation while the animal was left overnight at room temperature to allow the Microfil to cure completely (**Appendix B**).

Following the overnight curing period, the gracilis muscles from both limbs were carefully dissected and gently placed between two cover slips to maintain in situ orientation during the dehydration process. The muscles were dehydrated by a graded series of ethanol solutions (25, 50, 75, 95 and 100%) for at least 12 hours for each alcohol dilution. Following dehydration the muscles were placed into 100% methyl salicylate for clearance (16). Once cleared, the muscle was oriented correctly, placed onto a slide, covered with permount, coverslipped, and given 48 hours to dry before imaging. See Chapter 4 materials and methods for imaging.

Figure 8 – A) Microfil cast ingredients (from left to right) MV-Diluent, HV-Diluent, MV-112, Curing agent. B) In order to prep the animal the hindlimbs were trimmed with clippers and then the hair was removed completely with depilatory cream to minimize hair contamination in the sample. C) An incision was cut in the apex of the heart and PE-100 catheter tubing was inserted into the apex and clamped into place with a vascular clamp. D) Following clearance the 20mL syringe was loaded with the Microfil cocktail for perfusion. E) In order to gain access to the heart the ribs and diaphragm were quickly cut through and clamped back with hemostats in order to open the chest cavity. F) Ankle ligation to prevent Microfil from casting the venous side through A-V shunts. G) Proper clearance of the gracilis muscle was checked before Microfil was perfused. H) After Microfil perfusion gracilis was checked to make sure perfusion penetration was adequate and that there was no venous

Results

Achieving the desired results required two different studies with multiple experiments. Preliminary testing of the Microfil yielded poor results (**Figure 9**). Further experimentation adjusting the viscosities by varying the amount of MV-122 and HV-Diluent (**Table 1 – Study 1**) also provided poor results (**Figure 10**). For study 2, the ratios of diluent and MV-122 were kept at a constant 5:4 ratio,

Figure 9 – Preliminary Microfil yielded poor results as the filling is incomplete and spotty. This sample is of the right anterior near the saphenous artery.

Study 1		1	2	3	4	5
	MV-122 (ml)	1.5	2.0	2.5	3.0	3.5
	HV-Diluent (ml)	3.0	2.5	2.0	1.5	1.0
Study 2	MV-122 (ml)	2.0	2.0	2.0		
	HV-Diluent (ml)	2.5	1.25	0		
	MV-Diluent (ml)	0	1.25	2.5		

Table 1 – Studies with Microfil casting compound ratios. Study 1 was unsuccessful as adjusting the ratios between the MV-122 and Diluent ruins the compound. Study 2 was successful; it maintains the 5:4 ratio of Diluent to MV-122 while also varying the viscosity. Ultimately, experiment 2 of study 2 provided the wanted results.

while the viscosity was adjusted by mixing MV-diluent with HV-Diluent (**Table 1** – Study 2). Experiment 2 provided the desired results: solid casting with no bubbles, which only penetrated to the capillary level (**Figure 11**).

Figure 10 – A) Poor results from the left anterior of study 1-experiment 1 near the saphenous artery. B) Poor results from the left anterior of study 1-experiment 5 in the center of the gracilis muscle.

Figure 11 – Control animal B. A) Left leg anterior gracilis. B) Right leg anterior gracilis.

Discussion

Results from the new Microfil casting procedure were a great improvement over previous India ink vascular casts. **Figure 12** displays a successful India ink fill of the anterior gracilis. From **Figure 12**, it is clear that both the arterial and venous side of the circulation is filled. The India ink completely fills the arterial side, the capillaries (which look like strands of hair in the image) and then the

Figure 12 – A successful India ink fill casts both the arterial and venous side of the vascular system.

venous side. In order to use this sample for measurement one must visually isolate the arterial side from the venous side before proceeding. The interest for our lab lies in the arterial side of the circulation because that's where blood flow is determined and where vascular remodeling predominantly occurs following an occlusion (19); the venous side cast just adds confusion during morphology measurement. Although it is possible to discern arterial from venous by looking at

relative size of the vessels (the venous side is larger than the arterial side) mistakes are still made and often smaller arterial trees are missed due to the complexity of the images. Arteries and veins run parallel to each other and intertwine along their paths, causing difficulties when trying to differentiate between the two. The whole process would be easier if the vascular side wasn't casted. My final Microfil vascular casts (**Figure 11**) show that it is possible to cast the arterial side of the anterior gracilis using a ventricular catheterization. It is clear from **Figure 13** that the Microfil penetrates right up to the capillaries on the arterial side and then stops. Further, only the arterial side of the saphenous and profunda femoris is filled, which indicates that no Microfil was able to transition to the venous side through the A-V shunts in the lower leg.

As stated earlier, Microfil is a polymerizing silicone rubber compound that comes in three parts that must be mixed to create the injectable compound. This

Figure 13 – Magnification of Figure 11A to show penetration of Microfil at capillary level.

eliminates the other issues encountered with India ink as once Microfil has cured, it will not leak and the preparation involved is more straight-forward, requiring only mixing without the need to dialyze, filter, and purify (**Table 2**). All of the control Microfil samples can be found in **Appendix A**. It is important to note that

	Microfil	India Ink
High Contrast	Yes	Yes
Price (16 oz)	\$125	\$8
Preparation	Vortex components	Dialyze, filter and purify
Adjustable Viscosity	Yes	No
Curable	Yes	No
Radiopaque	Yes	No

Table 2 – Tabular comparison of Microfil versus India ink

although the same protocol was followed for every vascular cast, there are still variable results in the samples. This is likely due to the variability of catheter placement into the apex of the left ventricle during the ventricular catheterization. There is a very limited window of time to correctly cut the apex of the heart and clamp the catheter in place. If it is placed incorrectly it is difficult to shut off the syringe pump and reposition the catheter without damaging the tissue. There is also a risk of blood clotting, which will impede blood clearance and penetration of the casting agent into all arterial branches of the vasculature. Poor catheter position, damaged heart tissue, and blood clotting can all be factors that determine a good cast from a poor cast (**Appendix A** – replicate A versus replicate B).

To develop the Microfil casting protocol I began with a 4:5 ratio of Microfil MV-122 and HV-Diluent (**Figure 8A**), which was then catalyzed with 5% by volume of the curing agent. These values were taken from the Microfil manual

and also used by Lash and Marxen in their research groups (27, 33). Using these values I began my preliminary ventricular catheterizations and the results (**Figure 9**) were poor, showing incomplete and spotty filling. The initial conclusion was that the poor casting was due to an error in technique with the ventricular catheterizations, so more India ink casts were performed to compare to previous results. Once correct technique was established, more Microfil casts were performed with similar results. Efforts were then focused on varying the flow rate to achieve the desired results; the idea being that if the Microfil was too viscous, increase the force of injection. The flow rate was raised to 5ml/min (ink fills were performed at 3ml/min), with more poor results; any higher rate resulted in a pressure build-up that would cause the syringe pump to malfunction and sometimes catheter ejection from the heart. The next modification was to decrease the viscosity of the Microfil cocktail by adding less of the HV-Diluent and more MV-122. I experimented with a change in the HV-diluent to dye ratio in order to vary the viscosities and find a mixture that would allow for maximum penetration while also preventing venous return (**Table 1**); however, this approach yielded poor results, (**Figure 10**). The conclusion was that although the viscosities of the mixtures were different, the ideal ratio between the mixtures was changing and, therefore, the Microfil was unable to mix properly when vortexed, which yielded poor vascular casts.

A batch of the normal viscosity diluent—MV-Diluent, was ordered and used to mix with the high-viscosity diluent in a new attempt to alter the viscosity, while also still preserving the necessary ratios. This approach was markedly

more successful, which led to discovering the best mixture for maximum arterial penetration and minimal venous return during vascular casting (**Figure 11**). However, although the Microfil was stopping at the capillary level, it was still not viscous enough to be stopped at the artery level and so the Microfil would perfuse down into the lower leg and across the A-V shunts. This could be seen under the microscope as the Microfil would stop at the capillaries of the gracilis but would still return on the venous side of the femoral and saphenous arteries, causing backfill into the venous circulation of the gracilis. The solution to this problem was to tie a tourniquet around the ankle of the mouse to prevent the Microfil from entering the a-v shunt (**Figure 8F**). This was successful, with slight modification. Initially the tourniquets were tied after the vasodilator cocktail but before the Microfil, which caused a build-up of fluid pressure in the lower leg. This pressure build-up led to a reversal of the pressure gradient when the syringe pump was stopped and caused the Microfil to be pushed back and un-fill the artery. This problem was addressed by starting the Microfil casting with no tourniquet on the ankle and applying the tourniquet a few second after the Microfil passed the tourniquet site on the ankle. At this point the Microfil flow rate had been lowered to .5ml per minute. This decrease allowed for easier visualization under the microscope as well as providing ample time to perform the tourniquets.

Multiple variations of Microfil were tested before the ideal viscosity (keeping the ratio between diluent and MV-122 constant) was found. Even then, protocol adjustments needed to be made to accommodate the venous filling from

the a-v shunts. It is also important to note that the India ink casting protocol perfused 5ml of ink, even though a mouse only has ~2ml of blood volume. I had much better results when setting the pump to only deliver 1ml and visually inspecting the casting progress in the gracilis muscle. If the cast was complete, the Microfil perfusion was stopped. If the cast was incomplete, the pump was continued until adequate casting had been achieved. Performing vascular casts with Microfil was successful and future work in this area could test varying the ratios of the different diluents depending on the research needed. The new protocol calls for a 50/50 mixture of high-viscosity and normal viscosity diluent for the results shown. However, if further penetration of the capillaries or less penetration of the arterioles was desired, a lower or higher viscosity Microfil cocktail may be necessary, respectively.

Chapter 3 – Development of an Image Morphometry Macro

Introduction

Once the casted tissue is prepared (chapter 1) and imaged (chapter 4), morphological analysis is necessary. Important morphological parameters include: vessel length, vessel diameter, branch angle, vessel order, and vessel quantity. To manually measure these features is a laborious task; this entire project consisted of 32 samples requiring morphological analysis, with the samples averaging around 1000 vessels per sample (**Appendix H**). The first step in the morphological analysis is to order each vessel in each sample, followed by each morphological measurement. So, to obtain data on length, diameter, and branch angle requires 96,000 measurements, plus the time it takes to order each sample. The other issue with manual measurement is the variability in human measurement. Any type of automation or efficiency brought to this process that could decrease time and/or increase the consistency with measurement would be beneficial.

The general challenge with automatic determinations of vessel architecture is determining “vessel” from “non-vessel”. An algorithm needs to be written and “trained” with numerous samples. Then the vascular system can be broken up into individual segments and functions can be written to consistently measure the different morphology.

Two areas where automatic measurement and image analysis are being pursued are ophthalmology and neuroscience—where the morphology of retinal blood vessels and neuron axons, respectively, are important (5, 8). Interest in

measuring retinal vessel architecture stems from the fact that it is non-invasive and has been correlated with cardiovascular and cerebrovascular disease (8, 28). Retinal vessel measurement faces similar issues as our lab with manual quantification and measurements being slow and biased by the observer (8). Algorithms can be used to automatically determine arteriolar diameters (8) by fitting a vessel intensity profile to a double Gaussain function by non-linear regression, and using an edge detection algorithm. This model, created by Sobel, involves using a sliding linear regression filter to determine points of maximum intensity variation to determine “vessel” from “non-vessel” (8). Sophisticated algorithms require many samples to be tested in order to become more precise with each iteration. Chapman’s group found that even their most consistent and repeatable algorithms yielded measurements significantly greater than manual measurements (8). Even though the results between automatic and manual were different, there is still value in the consistency with which an automated program can make measurements. Ideally, a full automation strategy would be designed to acquire the necessary morphology measurements, however, due to the skillset and amount of samples necessary, a different avenue from full automation was pursued.

Our interest in microvascular topology requires manual measurements of diameter, length, branch angles, vessel order, and vessel quantity for input into a computational model for calculating hemodynamic conditions. Each of these morphology measurements must be evaluated in hundreds of vessels using morphometry software, such as ImageJ. This effort requires tracing thousands of

vessels manually, plus drawing the length and diameter of segments to obtain each of the morphological measurements; this process requires considerable time, even for those experienced with the technique—to order and measure the lengths and diameters of a sample would take about 2-3 hours, depending on the number of vessels. The goal for this portion of the research was to improve the efficiency of the manual tracing by developing a process to semi-automate the morphological analysis by combining the diameter and length measurements into one process instead of two.

Materials and Methods

Design Criteria

One of the goals of this thesis was to increase the efficiency of the morphology measurements. One of the inefficiencies I pinpointed was in the manual data collection. Ideally, a fully automated data collection program would solve this issue. However, full automation was not only outside of my skillset, but also would require approximately 100 samples to distinguish “vessel” from “not-vessel”; so instead, I opted to simplify the problem significantly. The objective for this aim of the thesis was to gather two diameter measurements and two length measurements, average the diameters and lengths independently, and record both the length and diameter of a vessel when the user defined the vessel with 4 mouse clicks. This, theoretically, would save time (since the user doesn’t have to measure the diameter and length separately), while also being a suitable replacement for manual measurement by outputting similar results or even

increasing the accuracy of the data collected (since two diameter/length measurements would be averaged, instead of making one measurement).

Vessel Morphometry Procedure

The automation program was written using ImageJ and the macro editor. To reiterate, the goal for the program was to calculate the length and diameter of a vessel by drawing a quadrilateral to approximate the shape of the vessel segment (**Figure 14**). This is accomplished with four user-defined points, which create the quadrilateral and then output an average vessel diameter (based on the average of H_{1-3} and $2-4$) and vessel length (L_{1-2} and $3-4$) (**Figure 14**). To define a vessel, 4 points are indicated to outline the vessel by hovering the mouse over each point and selecting the “a” key. The points must be selected in the correct order or the program will output incorrect measurements. Any mistakes can be reversed by selecting “y” to erase the previous selections. From the 4-points, the

Figure 14 – General idea for how the automation program would work. The user would click the 4 points in order to identify a given vessel; the program would then draw a quadrilateral and measure the length and breadth by averaging the distance between 1-2 and 3-4 and the average distance between 1-3 and 2-4, respectively.

macro creates a quadrilateral, whose length and height dimensions are automatically calculated and output into an ImageJ log file, which can be saved and exported into Excel (**Appendix D**). The code used to carry out these measurements can be found in **Appendix C**.

Automation Validation

As a simple test to test my macro, I drew 3 rectangles (**Figure 15**) and measured them manually and with the macro in ImageJ. The results (**Table 3**) show that the macro is calibrated (calibration is built into the macro so re-calibration for every image is unnecessary—see manual protocol in **Appendix L**), measuring correctly (**Table 3**) and ready to be tested with a more robust

Figure 15 – 3 rectangles measured manually and with the macro to test functionality.

	Length			Diameter		
	Manual	Macro	% Difference	Manual	Macro	% Difference
1	655.652	656.522	0.13%	133.913	131.304	1.95%
2	787.826	789.565	0.22%	66.087	66.957	1.32%
3	986.087	986.087	0.00%	133.913	132.174	1.30%

Table 3 – Automation validation results

comparison on vascular casted samples. Next, four gracilis muscle samples were chosen (ECLNI, ECRNI, FCLNI and FCRNI) and were measured with the macro and manually to examine the accuracy of the program when measuring blood vessels instead of rectangles. Those four samples were chosen because they had the highest number of blood vessels, which would yield a more powerful comparison.

JMP was used for the statistical analysis to determine if the macro could substitute for manual measurement of lengths and diameters. Because there were multiple orders and therefore multiple measurements per sample a repeated measures analysis was performed; statistical significance ($p < 0.05$) between each condition was assessed using the Tukey-Kramer test, and a block was placed on the mouse to eliminate mouse-to-mouse variability within the results (**Appendix E**).

Results

The macro-obtained measurements were compared to the manually obtained measurements to examine if the macro could be used as an acceptable substitute to manual measurement. Average lengths and diameters from samples ECLNI, ECRNI, FCLNI and FCRNI were used to calculate the least square means for each order and then the least square means were compared for each order. The results show no significant difference between macro and manual measurement except between order 5 length values (**Figure 16 and 17**).

Figure 16 – Macro versus manual measurements for length showed a significant difference for order 5 vessels.

Figure 17 – Macro versus Manual measurements for diameter showed no significant differences.

Discussion

The results showed that that the only significant difference between the macro and manual measurement came from measuring order 5 lengths. The reason for this difference stems from the way that the macro is estimating values. When a vessel is straight (**Figure 14**) using a quadrilateral to approximate the

vessel works well and you can expect to get an accurate value for the length of the vessel; however, when a vessel becomes more tortuous (**Figure 18**), a quadrilateral estimation isn't as accurate. I expected this would only be a problem with vessel lengths as the diameters are always a straight measurement. When looking at the samples it seemed as tortuosity went down the higher you went in vessel order, yet surprisingly, the only difference came in order 5 vessels, and I think the only reason for that is the sample size, which was 35 order 5 vessels. Since the program is measuring straight lines for curving vessels, the length measurements are always going to be shorter than actual measurements, but

Figure 18 – Using the automation tool will approximate a vessels length if it is curved. The program output a length of 76.52 μm , while the manual measurement output 123.01 μm , which is a 37.79% difference.

statistically, this only makes a difference for order 5 vessels. Therefore, if this program is used to substitute manual measurements, order 5 lengths should still be measured manually.

The major advantage to this program is one of organization, which leads to efficiency. When four points are selected and a rectangle is drawn and

measured, the rectangle outlining the vessel remains as a marker for which vessels have been measured already (**Figure 14**). This doesn't happen when using the manual measurement technique, so remembering which vessels have been measured becomes challenging. The macro also groups length and diameter into one measurement output, rather than manually measuring the length and the diameter in two different measurements.

Conversely, one difficulty with the macro is still the time it requires to make a measurement. Hovering over four different points and clicking a button on a keyboard to define thousands of vessels still takes a lot of time. The macro is a step in the right direction, but there are still improvements to be made that could save more time. Another issue is measuring the lengths of level 5 vessels. Since there are a low amount of level 5 vessels per sample an easy solution is to switch to manual measurement. Human error is another issue, since both methods require a user to define a vessel; some variance will always be present. The percent differences seen in **Table 2** demonstrate that even with simple samples it's hard to define the same start and end for the manual and macro techniques; however, this small of a variance is unlikely to have any effect on the morphometry.

The initial logic for the development of the macro required the macro to wait for 4 clicks from the user, sort the points, draw the correct quadrilateral, and then make the calculations and output. However, limited programming abilities required adjusting the initial logic while still obtaining the desired output. For example, using the "getCursorLoc" command (meant to pick points when user

clicked the left mouse button) was really hard to work with and picked up left and right clicks, click releases, and rollovers. Searching online discussion forums I came across the use of a “hot key” to label points instead of a mouse click; implementing this method eliminated the “getCursorLoc” issues, and instead of 4 mouse clicks, required the user to hover over the point of interest and press the letter “a” to define a point. Problems also arose when trying to create a “sort” function to order input selections from the user correctly, but was solved by requiring the user to select their points in the following order: upper left, upper right, lower left, and then lower right. The input always comes in the same order so the calculations are performed correctly. Output was another issue; initially the program output the values as one single column and exporting it to excel placed all of the data into cell A1 instead of individual cells. Updating the code to a new multi-column format and adding commas after each data point so that excel could use them as a delimiter allowed for excel import into separate cells, in 7 columns. Slight deviations were taken from the initial design criteria; however, the final macro accomplished the goal of recording length and diameter values with one measurement.

This program is a good starting point to progress away from manual measurement but it is still relatively basic, and numerous features could be added to optimize morphological measurement. Currently, it gathers data from four clicks to output the same length and diameter measurements as the manual method, all things being equal. Ideally, the program would gather all of the desired morphological measurements. As stated earlier, an artificial intelligence

algorithm could solve this problem by scanning the sample and through pixel recognition decide whether a pixel is “vessel” or “not vessel”. This would be done throughout the image until the program has a data-driven image of the vascular system, which it would use to compute the morphology values. This level of automation is achievable but requires intensive knowledge of algorithm development in image recognition and a high number of samples to test for algorithm augmentation. A more feasible next step for our lab would be to integrate branch angle measurement into the macro. A simple addition would use the ImageJ angle measurement tool and mark which angles have already been measured. A more elegant addition would store every defined vessel as its own element, then the user could select two adjacent elements and the program would calculate the branch angle between them.

Chapter 4 – The Impact of Collateral Enlargement on Transverse Arteriolar Tree Structure

Introduction

After creating a successful vascular casting protocol using Microfil and a measurement automation program, the next step was to gather morphological data on a remodeled vascular network following arterial occlusion. PAD is caused by insufficient blood flow to a tissue, which is usually induced by atherosclerotic lesions that produce increased resistance and decreased blood flow to the distal tissue, sometimes completely occluding an artery (20, 37). PAD has many factors that can contribute to the pathophysiology, which is why a ligated model was used to replicate an arterial occlusion in order to examine the impact collateral enlargement has on transverse arteriolar tree structure.

The goal of this model is to induce blood flow changes and repair processes that we can relate to PAD. Blood that previously flowed through the femoral and saphenous arteries will mainly be rerouted through the deep femoral and profunda femoris (34). Essentially, there are no more large arteries to feed the distal hindlimb following ligation; this causes an increase in flow as oxygen and other nutrients are rerouted through the gracilis collateral flow path to reach the lower limb. The downstream tissues experience hypoxia and inflammation in the lower leg, which promote angiogenesis (34). The ligation also causes an increase in wall shear stress through the collateral vessels from the elevated blood flow, which will result in the activation of endothelial nitric oxide synthase (eNOS) to produce NO to cause vasodilation (25). The immediate effects of the

increased shear stress will cause vasodilation due to the blood flow divergence, while the chronic increase of shear stress will induce arteriogenesis (4, 43, 44). Therefore, we can expect to see the collateral arteries that feed the gracilis muscle to outwardly remodel in order to provide the blood flow needed for recovery of the hindlimb (6, 7). The next step becomes investigating if the increased flow and shear stress in the collateral results in a change in the transverse arteriole (vessel order 4 and 5) networks branching off. This is important for a few reasons: the presence of collaterals is a decent indication of a patient with ischemic disease so stimulating arteriogenesis in these collaterals could be used as a therapy for PAD. However, it is unknown how arteriogenesis affects the microcirculation in the muscle housing the enlarged collateral and since the microcirculation is critical to nutrient and waste exchange, changes could affect muscle functionality. On one hand the increased flow could lead to architecture and vessel expansion, and on the other, the increase of pressure could lead to regression. This study could help determine if stimulating arteriogenesis could be a concern because of the possibility of compromised flow, and therefore, functionality, in the collateral-containing muscle.

Materials and Methods

Ligation Surgery

All protocols were approved by the Institutional Animal Care and Use Committee. Eight C57BL/6 mice were used as the experimental group and had a ligation tied around their left femoral artery just distal to the profunda femoris, while a sham surgery was performed on their right leg. The surgeries were

performed under aseptic conditions and using isoflurane anesthesia. Prior to the procedure each animal was prepared by removing the hair over the surgical area with clippers and depilatory cream and then disinfecting the area with Nolvasan. The mice were maintained at $35\pm 1.0^{\circ}\text{C}$ using a rectal-temperature controlled heating pad. The initial incision was made on the medial aspect of the hindlimb, directly above the femoral neurovascular bundle, and was extended upwards to the abdominal wall and downwards towards the origin of the saphenous artery, making sure to keep the incision over the neurovascular bundle. Sterile saline solution was used to irrigate the tissue and prevent muscle desiccation. Connective tissue was blunt dissected to increase exposure. Following connective tissue removal the epigastric fat pad was removed by heat cautery to expose a site for the ligation just downstream to the profunda femoris. At the chosen spot for ligation the nerve was carefully blunt dissected away from the femoral artery, and then the vein was separated from the femoral artery in a similar fashion. To ligate, 6.0 silk suture was used; following ligation, the skin incision was closed with 7.0 polypropylene suture.

The sham surgery procedure included an incision above the femoral neurovascular bundle. Sterile saline was used to irrigate and prevent tissue desiccation while connective tissue was blunt dissected. Special care was taken not to contact the neurovascular bundle in order to simulate every aspect of the ligation except the vascular insult. Finally, the skin incision was closed with 7.0 polypropylene suture. The mouse then received a subcutaneous injection of buprenorphine (0.075 mg/kg) and was allowed to recover on a hot pad until

ambulatory (**Appendix K**). Mice were then returned to the Cal Poly vivarium for 2 weeks before the vascular casting procedure.

The Cal Poly vivarium is maintained at $73\pm 5.0^{\circ}\text{F}$ and lights are on a 12 hour light-dark cycle. Each micro-isolator cage houses no more than 4 mice and is changed at least once per week. Mice are provided food and water ad libitum, in addition to the following enrichments: mouse house, nesting material, and a plastic tube. During cage changes, lab members wore isolation outfits that included: full gown, gloves, face mask, bonnet, and booties to preserve aseptic conditions. Further, mice were monitored on a daily basis, during which their health and any irregularities were recorded and/or addressed as necessary.

Imaging

Sample images were taken using a QImaging Retiga EXi camera at 4x magnification on an Olympus BX41 microscope using bright field microscopy (**Figure 19**). QCapture Pro was used to capture the images after adjusting for maximum contrast. Because of the size of the gracilis and the level of resolution needed for morphological measurements, multiple images of the sample were taken and then stitched together into a photomontage using the photomerge function in Adobe Photoshop CS3 (**Appendix M**).

Figure 19 – Bright field microscopy setup. A) QImaging Retiga EXi camera. B) Olympus BX41 microscope.

Manual Measurement using ImageJ

Before measuring morphological parameters in a sample, branching order was determined using the Strahler method. Strahler ordering begins by defining the terminal branches of a given circulation. The terminal branches were assigned 1. When two vessels of equal order come together (i.e. order n) at a node or branch point, the parent vessel is assigned the next highest order, i.e. $n+1$ (**Figure 20A**). When two vessels of unequal order come together at a node or branch point (i.e. order n and $n-2$), the parent vessel is labeled order n (**Figure 20B**). Once all vessels were ordered morphological measurements were obtained using the macro (described in chapter 3) or by manual methods

(**Appendix L**) and vessel counts were gathered from the total diameter measurements.

Figure 20 – A) When two vessels of order n meet, the parent vessel is defined as order $n+1$. B) When a vessel of order n and a vessel of order $n-1$ meet, the parent vessel is labeled an order n .

Statistical Analysis

JMP was the software packaged used for the statistical analysis. The experiment aimed to answer two questions: 1) does the sham surgery accurately reflect the true control, and 2) is there a difference in vessel morphology in the transverse arteriolar trees between the ligated and non-ligated samples? The answer to the first question determined whether the ligated limbs were compared to the sham limbs or to the true control limbs i.e. the samples from Chapter 2, where only a vascular cast was performed without surgical intervention. Before the statistical analysis, each sample was organized and measured. Each sample was ordered (**Figure 20**) and if a sample didn't have order 5 vessels it was labeled as a poor cast and the data was excluded from the statistical analysis. Of

the 32 total samples 9 were labeled as poor fills. Once the sample was ordered, each ordered vessel's length and diameter was recorded and averaged so that for a given sample there was an average length and diameter for each individual order. Order 6 diameter values were taken from 5 measurements along the longitudinal arteriole, from the saphenous artery to the profunda femoris, which were then averaged. Order 6 length measurements were not taken. Because there were multiple orders and therefore multiple measurements per sample a repeated measures analysis was performed in JMP (**Appendix J**); statistical significance ($p < 0.05$) between each condition was assessed using the Tukey-Kramer test (**Appendix I**). When comparing sham legs to ligated legs, a block was placed on the mouse to eliminate mouse-to-mouse variability within the results. Statistical significance of vessel counts ($p < 0.05$) between the two treatments across each order was assessed using t-tests (**Appendix N**).

Results

From the eight ligated mice, replicates C and H were labeled as a poor casts and were excluded from analysis. From the true controls, replicates A, C and the right leg from H were excluded (**Appendix F**). **Figure 21** depicts a successful vascular cast on a ligated animal.

Figure 21 – Ligated animal E. A) Right leg – Sham surgery. B) Left leg – Ligated.

Average lengths and diameters from the samples (**Appendix G**) were used to calculate the least square means for each order. The right leg values from the ligated animals (sham) were compared to the right leg values from the animals only casted using Microfil (true control) to examine if the sham surgery was an appropriate estimation of normal. Comparing control and sham found a significant difference in the order 5 for length measurements (**Figure 22A**). No significant differences were found between sham and control for diameter measurements (**Figure 22B**). Comparing vessel counts between treatments across each order showed no significant differences (**Figure 23A**); there was also no significant differences between sham and ligated legs for length measurements (**Figure 23B**). Comparing sham and ligated legs for diameter

measurements found a significant difference in the order 6 measurements (Figure 23C).

Figure 22 – Right legs (sham) of the ligated mice compared to the right legs of the control mice (Microfil only). A) Length measurements – significance found between control and sham at order 5 only. B) Diameter measurements – no significance found between any orders

Figure 23 – Right legs of ligated mice (sham) compared to the left legs of ligated mice (ligated). A) Vessel counts – no significance found. B) Length measurements – no significance found. C) Diameter measurements – significance found between sham and ligated legs at order 6 only.

Discussion

Many factors contribute to the pathophysiology of PAD (46); therefore, a mouse model was used to model one aspect of the pathology: an arterial occlusion. The occlusion offered an increase in blood flow to the collateral circuit in order to examine whether arteriogenesis impacts the transverse arteriole architecture branching from the collateral. This could allow a better understanding into how therapies to stimulate arteriogenesis would impact the microvasculature and tissue function.

Comparing the sham surgery versus the right leg of the true controls yielded a significant difference between the lengths of the order 5 vessels, with the sham lengths being significantly greater than the control lengths. Although we see this difference in the order 5 lengths of the sham and control samples, this alone is not enough to say that the sham surgery cannot be used to estimate the true control. Poiseuille's law states that, all else being equal, an increase in length will result in a decrease in volumetric flow. However, the length is not as powerful of a factor as the radius of the vessel and there aren't any significant differences in the order 5 or even the order 6 diameters. This difference in length is likely not a reflection of differences in the underlying biology, but rather a reflection of insufficient replicates and therefore the sham can be used as a control and simply opening the animal for surgery will not cause any significant vessel remodeling for our purposes.

Comparing the sham leg to the ligated leg yielded a significant difference between the diameters of the order 6 vessels. To reiterate, the order 6 vessels

refer to the collateral arteriole that runs from the profunda femoris to the saphenous artery. Arteriogenesis in this vessel is well-described (16), and is induced by the elevated flow (shear stress) that is resultant of an increased pressure gradient across the collateral vessel following ligation. Increased shear stress is a trigger for increases in arterial diameter and outward remodeling of the arterial wall will occur to normalize the hemodynamic shear stress (4, 19, 38). So, one reason why the elevated flow didn't alter transverse arteriolar architecture is because the increased hemodynamic shear stress caused by the ligation had dissipated by the time the transverse arteriolar level was reached due to the arteriogenesis seen in the collaterals. Another explanation could be due to cellular communication between gap junctions that override the hemodynamic stimuli and cause the network to remain relatively unchanged. Therefore, it's possible that stimulating arteriogenesis in a clinical environment should not cause any negative effects in the collateral-containing muscle. However, this observation is potentially limited by the differences between the ligation model and a patient.

The femoral artery ligation model is a simplified model of a patient with PAD (it only simulates an occlusion), which is why it's important to understand the differences between the model and PAD. One of the main differences is the cause for the conditions, which are significantly different. When the femoral artery is ligated, the arterial occlusion occurs quickly, as the suture is pulled taut. In the case of a patient with PAD, occlusion occurs as a result of atherosclerosis, which occurs gradually as the build-up of plaque causes the narrowing of the

arteries over many years (46), however, when a thrombus forms, partial occlusion transitions to complete occlusion rapidly. In patients with PAD, complete occlusion of arteries often results in limb necrosis without proper treatment (10-40% have primary amputations and there is a mortality rate of 40-70% in 5 years) (14). Further, humans with PAD typically present with other co-morbidities like heart disease or obesity and have a gradual arterial narrowing due to atherosclerosis at multiple vascular sites throughout the body (46). It is likely that these co-morbidities explain the lack of revascularization. This results in a slower impairment of blood flow and progression of hypoxia and could alter the mechanisms for vascular repair (46). Conversely, in the mouse, hindlimb revascularization occurs and the mouse is able to recover over a period of 2-4 weeks sans intervention or treatment (34). The ligation model results in an arterial occlusion at one point, so, while both PAD and the model induce an occlusion in the extremities and hindlimb, respectively, the mechanisms are different. It is important to be aware of these differences as one interprets experimental results and makes conclusions regarding the process of revascularization and how it may influence recovery of vascular function following arterial occlusion when using a mouse model.

Interesting future experiments could gather branch angle measurements to add to the analysis. Further, flow measurements could be taken for the whole muscle to examine the impact of architecture versus reactivity or experiments could examine the impact architecture has on vasodilation. Future applications of this research could try to address one of the major concerns with therapeutic

angiogenesis: tumor-like microcirculation. Previous experiments delivering basic FGF or VEGF developed stable vascular networks; however, they soon began to resemble immature tumor vasculature (11, 14). It's important to understand why that happened by better understanding the network repair process. This study examined the repair process in a simple animal model, so the next step would either be to examine different muscles in a hypoxic area or to deliver growth factors and see how the network changes, which could eventually lead to finding what morphology values differentiate a healthy network from a tumor network and how a network can be prevented from diverging from stable to tumor-like. Other experiments would examine the role of gap junction communication in network remodeling by performing similar experiments in connexin knockout mice.

Before proceeding with future experiments it would be advantageous to investigate new methods to make data collection more efficient. Measuring the samples requires a high level of resolution for order 1 vessels; 15-25 photomicrographs must be captured and then montaged, sometimes manually. Ideally, a high-resolution camera would capture a full sample image with enough detail that measurement of order 1 vessels is possible. High resolution cameras capable of this task exist but are available in the \$40,000 range. A more plausible solution would be to capture up-close representative images. This would require identification of the large transverse arteriole trees that reach order 5 vessels; these arteriole trees would be ordered and measured and would give a sufficient representation of the vascular network. Of the samples prepared there were

between two and five of these arteriole trees per sample, on average. Capturing only the large transverse trees would decrease the images needed from between 15 and 25 for an entire sample to between 5 and 15 to capture the chosen trees.

Chapter 5 - Discussion

Previous studies have revealed the complexity of vascular remodeling (19, 31, 38) and relatively simple treatments in clinical trials have yielded less than optimal results (11, 14). In order for therapeutic angiogenesis to be successful, there needs to be a more comprehensive understanding of the vascular growth process to understand why therapeutic angiogenesis was unsuccessful in clinical trials. A more complete understanding of the effects of revascularization on vascular network topology could provide information to devise more efficacious therapeutic angiogenesis treatments for patients with PAD.

The overall objectives of this study were to establish procedures to increase the efficiency of microvascular network morphology and use these procedures to investigate microvascular remodeling following arteriogenesis. The aims for the thesis were as follows:

- 1) Create a vascular casting protocol to only cast the arterial side of the microcirculation.
- 2) Decrease the time to measure microvascular morphology through some form of automation.
- 3) Determine the impact of collateral artery enlargement on the topology of arteriolar networks that branch from the collateral artery.

Through Aim 1, I developed a vascular casting protocol to only cast the arterial side of the microcirculation with the commercially available casting compound Microfil. This protocol required an adjustment of the Microfil viscosity. The ideal mixture for casting the arterial side was a ratio of X MV-122 to Y of

both the high and normal viscosity diluent (as long as Microfil was prevented from entering the lower leg A-V shunt). Because this was successful, the venous side no longer complicated the image; this allowed for a more thorough vessel identification of arterial trees, trees that were previously lost among the confusion of India ink casting were now measurable. When ordering an India ink cast it is difficult to discern between arteries and veins, especially for lower ordered vessels. This leaves the user to select arterial trees that were easy to identify. This results in much lower vessel numbers than the Microfil casting technique. Since Microfil casting makes it much easier to identify arterial vessels, more vessels were measured (**Appendix H**) and, therefore, more time was required for data collection. Future modifications could order the entire sample and then only measure the arteriole trees that reach order 5 before connecting back to the longitudinal arteriole. This aim only examined the gracilis, but future work could adapt the protocol to include other muscles like the spinotrapezius, and it could even be adjusted to fill less of the arterial circulation, depending on the branching order of interest.

Through Aim 2 I decreased the time for morphological measurement of the anterior gracilis with a macro written in ImageJ. For this procedure, the vessel is defined with four clicks; the macro creates a quadrilateral with the points and then measures the two lengths and diameter values to output an average length and diameter for the selected vessel. Conversely, manual measurement requires separate length and diameter measurements. Surprisingly, results found that the macro's estimation of length was only significantly different for order 5 vessels,

where the sample size was small. Overall, the macro is a first step in making morphometry measurements more efficient. The next iteration of the process would add a vessel angle measurement to the macro, such that all three measurements are bundled into one step. One of the unforeseen side effects of the new vascular casting protocol was that because the venous vasculature was un-casted, arterial vasculature was much easier to identify, which increased vessel numbers significantly and therefore inadvertently increased the measurement time. To maintain efficiency, focus should be kept on measurement of well-casted vascular trees; meaning, after ordering a sample, measurements should only be made in vascular trees that reach order 5 before connecting back to the longitudinal arteriole.

For the 3rd and final aim, I used the casting procedure and morphology macro, developed in Aims 1 & 2, to compare transverse arteriolar networks branching from an enlarged collateral to transverse arteriolar networks branching from a non-enlarged collateral. As expected the collateral arteriole (order 6) did outwardly remodel. We expected that the increased flow and shear stress in the collateral would impact the transverse arteriole networks branching from the collateral. Surprisingly, there was no significant difference for any diameters, lengths, or vessel numbers for orders 1 – 5, indicating that the networks aren't significantly changing. This could be due to the lack of hemodynamic shear stress (absorbed by the outward remodeling of the collateral arteries), which would cause outward remodeling or it could be due to cell-to-cell communication through gap junctions overriding the hemodynamic stimuli.

It is important to note that using Microfil as a casting agent doesn't completely eliminate issues arising with discerning vessels for measurement. Complex vessel branching patterns necessitate subjective measurement of multi-directional networks that frequently overlap and intertwine. The gracilis muscle is also three-dimensional, so any vessels in the z-direction may not be measured accurately, however, since the gracilis is relatively flat, this likely didn't impact on results. Another issue identifying vessels stems from inconsistent dissecting of the gracilis muscle that leaves some samples with other muscles that underlie the gracilis and add layers of vessels that are unwanted.

Overall, pairing the new Microfil casting technique with the photomerge stitching functionality in Photoshop yields an image that shows the larger picture of vascular structure following an arterial occlusion. Furthermore, because of the high resolution of the images, one is able to make measurements of lower order vessels to gather data to explore changes in the vascular structure. The ligation model experiments demonstrated functionality of the new techniques and laid the groundwork for further experiments to be performed where vascular topology is examined rather than vessel cross-sections for the development of PAD treatments. Ultimately, more thorough analysis of microvascular network topology following arterial occlusion should assist in the development of successful therapeutic angiogenesis treatments for patients with PAD.

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Appendix A

The following are the true control samples. ACRNI is a full sample label where “AC” denotes control animal a, “R” denotes right leg, and “NI” denotes non-ischemic.

ACLNI

ACRNI

BCLNI

BCRNI

CCLNI

CCRNI

DCLNI

DCRNI

ECLNI

ECRNI

FCLNI

FCRNI

GCLNI

GCRNI

HCLNI

HCRNI

Appendix B – Microfil casting and dissection protocol

Date _____

Mouse Information

DOB:

Sex: _____

Tag: _____

Genotype/strain: _____

Cage: _____

Weight (g): _____

Materials

Non-Sterilize Dissection Instruments

- ___ 1. Forceps (1)
- ___ 2. Bone Scissors(1)
- ___ 3. Skin Scissors (1)
- ___ 4. Hemostats(1)
- ___ 5. Spring Scissors for Ventricle (1)
- ___ 6. Vascular clamp (1)

Obtained in surgery suite

7. Tape
8. 20mL Syringa (1)
9. Bench cover
10. Heating pad
11. Catheter/Stopcock/Blunt needle (PE-100)
12. Isoflurane Anesthetic
13. Gauze squares

Vasodilator Cocktail Preparation

14. Turn on water bath to 37°C
15. 400 μ L heparin
16. 1mL SNP (orange)
17. 600 μ L Adenosine (clear)
18. 38mL PBS solution
19. Thaw SNP and Adenosine
20. Add heparin, SNP, Adenosine, and PBS solution together in a 50mL conical
21. Place vasodilator cocktail in water bath

Microfil Cocktail Preparation

22. 2mL Microfil MV-122
23. 1.25mL Microfil HV-Diluent
24. 1.25mL Microfil MV-Diluent
25. Add MV-122, HV-Diluent & MV-Diluent into a 14mL conical vortex, place in bath

Procedure Preparation

26. Weigh animal
27. Obtain saline filled petri-dish, cotton swab, and instruments

Fixation

28. Anesthize mouse with Isoflurane
29. Heat up heat pad in microwave and wrap with bench cover when warm
30. Remove hindlimb hair on both legs by shaving
31. Tape animal down to heated bench cover
32. Separate skin from muscle from the abdomen to the top of the thoracic cavity
33. Fill 20mL syringe with warm Vaso D

Microfil

Initials

34. Cut through abdomen close to diaphragm
35. Quickly cut through the ribs and diaphragm to open chest cavity and clamp back with hemostats
36. Cut away excess tissue around the heart
37. Make a small incision in the apex of the heart
38. Insert catheter and clamp with vascular clamp and cut right atria
39. Inject Vaso D solution into animal approximately 20mL at 5mL/min x2
40. Add .2mL of curing agent to Microfil
41. Inject 1mL of Microfil Cocktail (.5mL/min)
42. Pause microfilm and ligate both ankles with silk suture once Microfil has passed the knee. Resume Microfil.
43. Repeat step 41 until satisfied with fill.
44. Remove mouse from heat pad and take out catheter (keep heart clamped)
45. Place mouse in bag (cover open wounds with saran wrap) and let sit overnight at room temp
46. Cover scope, turn off water bath, turn off oxygen, turn off Isoflurane, clean instruments

Dissection Date:

Date:

Non-Sterilized Dissection Instruments

- _____ 47. Fine Forceps (2)
 _____ 48. Forceps (1)
 _____ 49. Iris Scissors (1)
 _____ 50. Dissection scissors (1)

Obtained in surgery suite

51. Non-sterile saline
52. Cotton swab
53. Bench cover
54. Tape
55. Petri Dishes (2)
56. Cover Slips (2)
57. Disposable pipettes (1)
58. 25% ethanol
59. Dissect chosen muscles and place on cover slip and then in labeled petri dish of 25% ethanol
60. Cover scope and clean instruments

Notes:

Appendix C – Program code – Logic comments are displayed in green

```
var loc = newArray(0,0,0,0, 0,0,0,0);
var count = 0;
var tot = 1;

macro "Sample Tool [a]" {
    % Prints correct headers
    if (tot == 1 && count == 0) {
        print("Pol #,H1 (um),W1 (um),H2 (um),W2 (um),AvgH (um),AvgW (um)");
    }
    % gets x,y,z coordinates on cursor location when "a" is clicked
    getCursorLoc(x, y, z, flags);
    loc[count] = x;
    loc[count + 1] = y;
    makePoint(x, y);
    setForegroundColor(0, 0, 0);
    run("Draw");
    count = count + 2;

    if(count > 7) {
        calc();
        count = 0;
    }
}
% Allows user to undo a mistake by pressing "y"
macro "Clear [y]" {
    while (count > 0) {
        run("Undo");
        count -= 2;
    }
}
% Uses 8 user defined values from 4 locations to calculate and print calibrated height and width
function calc() {

    makePolygon(loc[0], loc[1], loc[2], loc[3], loc[6], loc[7], loc[4], loc[5]);
    setForegroundColor(0, 0, 0);
    run("Draw");

    h1 = loc[5] - loc[1];
    h2 = loc[7] - loc[3];
    w1 = loc[2] - loc[0];
    w2 = loc[6] - loc[4];

    aveH = (h1 + h2) / 2;
    aveW = (w1 + w2) / 2;

    print(tot + ", " + h1 / 0.575 + ", " + w1 / 0.575 + ", " + h2 / 0.575 + ", "
    + w2 / 0.575 + ", " + aveH / 0.575 + ", " + aveW / 0.575);

    tot++;
}
```

Appendix D – How to use Automation Macro Protocol

To use the tool, first move the automation tool file “automation tool.ijm” into ImageJ’s macros folder, most likely found at the path seen in figure A on the following page. Next, run ImageJ and click to install a new macro (B). Find “automation tool.ijm” and open it. Make sure the “point tool” is selected on the ImageJ tool bar and define boxes around a vessel by clicking in the correct order (C). Once every vessel has been defined and measured, open the text log and save it (D). Next, open excel and choose to open a file; find the saved text log from ImageJ (if the text log isn’t found change the file types in Open from “All Excel Files” to “All Files”) (E). Upon arrival to the text import wizard; make sure “delimited” is checked on the first page (F) and that “tab” is checked on the second page (G) and then click finish to get the results in an excel format (H).

Computer > VistaOS (C:) > Program Files > ImageJ > macros

ImageJ

File Edit Image Process Analyze Plugins Window Help

Macros Install... Ctrl+Shift+M

Shortcuts Run...

Utilities Edit...

New Startup Macros...

Compile and Run... Record...

3D Pencil Tool Options...

Analyze Paintbrush Tool Options...

Flood Fill Tool Options...

Set Drawing Color...

About Startup Macros...

Save As JPEG... [J]

Delimiters

☐ Tab

☐ Semicolon

☒ Comma

☐ Space

☐ Other:

Original data type

Choose the file type that best describes your data:

☒ Delimited - Characters such as commas or tabs separate each field.

☐ Fixed width - Fields are aligned in columns with spaces between each field.

Log

File Edit Font

Save As... Ctrl+S

Pol# H1 W1 H2 W2 AvgH AvgW

1,31.3043 um,5.2174 um,33.0435 um,3.4783 um,32.1739 um,4.3478 um

2,5.2174 um,60.8696 um,1.7391 um,53.9130 um,3.4783 um,57.3913 um

3,5.2174 um,113.0435 um,6.9565 um,118.2609 um,6.0870 um,115.6522 um

4,34.7826 um,3.4783 um,36.5217 um,5.2174 um,35.6522 um,4.3478 um

5,6.9565 um,86.9565 um,5.2174 um,86.9565 um,6.0870 um,86.9565 um

1

2

3

4

All Excel Files

All Files

All Excel Files

Excel Files

All Web Pages

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All Data Sources

Access Databases

Query Files

dBase Files

Microsoft Excel 4.0 Macros

Microsoft Excel 4.0 Workbooks

Worksheets

Workspaces

Templates

Add-Ins

Toolbars

SYLK Files

Data Interchange Format

Backup Files

OpenDocument Spreadsheet

	A	B	C	D	E	F	G
1	Pol#	H1 (um)	W1 (um)	H2 (um)	W2 (um)	AvgH (um)	AvgW (um)
2	1	31.3043	5.2174	33.0435	3.4783	32.1739	4.3478
3	2	5.2174	60.8696	1.7391	53.913	3.4783	57.3913
4	3	5.2174	113.0435	6.9565	118.2609	6.087	115.6522
5	4	34.7826	3.4783	36.5217	5.2174	35.6522	4.3478
6	5	6.9565	86.9565	5.2174	86.9565	6.087	86.9565

The steps to install and run the automation tool. A) The most common location of the ImageJ macros folder where the “automation tool.ijm” file should be copied. B) How to install a new macro. C) The correct click order to define a vessel for measurement. D) The log file of measurements that must be saved in order to export to excel. E) Select “all files” in order to open your log file in excel. F) Import screen 1 – make sure delimited is checked. G) Import screen 2 – make sure comma is checked. H) Example of final Excel output.

Appendix E – Macro versus Manual statistical comparison

Repeated measures analysis setup in JMP (this window can be accessed in JMP by going to Analyze>Fit Model in the toolbar) and Tukey-Kramer results.

The screenshot shows the JMP software interface. At the top is a data table with columns: Mouse, Leg, Treatment, Order, Length, and Diameter. Below the table is the 'Report: Fit Model - JMP' window. The 'Model Specification' tab is active. In the 'Select Columns' list, 'Length' and 'Diameter' are selected. In the 'Pick Role Variables' section, 'Length' is assigned to the Y role. The 'Personality' is set to 'Standard Least Squares', 'Emphasis' is 'Minimal Report', and 'Method' is 'REML (Recommended)'. The 'Construct Model Effects' section shows 'Mouse', 'Treatment', 'Mouse*Treatment& Random', 'Order', and 'Treatment*Order' in the list. The 'Degree' is set to 2. A dropdown menu is open for 'Attributes', showing 'Random Effect' and 'Response Surface Effect' options.

Least Sq Mean			A	Least Sq Mean			B
Level				Level			
Manual,5	A	26.437827		Manual,5	A	157.32165	
Auto,5	A	24.503213		Auto,5	B	125.56995	
Manual,4	B	20.156047		Manual,4	B	125.48410	
Auto,4	B	18.068120		Auto,4	B C	103.30397	
Manual,3	C	12.658170		Manual,3	C D	96.56617	
Auto,3	C D	11.493173		Manual,2	C D E	80.92198	
Manual,2	C D E	8.410013		Auto,3	C D E	80.67870	
Auto,2	D E	8.089149		Manual,1	D E	72.64188	
Auto,1	E	6.814805		Auto,2	E	68.93703	
Manual,1	E	6.655195		Auto,1	E	64.30079	

Levels not connected by same letter are significantly different.

Tukey-Kramer Results – A) Diameters B) Lengths

Appendix F – Ligated Animal Vascular Casts (NI – Sham Surgery, I – Ischemic/Ligated)

ALI

ARNI

BLI

BRNI

CLI

CRNI

DLI

DRNI

ELI

ERNI

FLI

FRNI

GLI

GRNI

HLI

HRNI

Appendix G – Averages of samples. Order 6 diameters were taken from 5 measurements along the longitudinal arteriole, from the saphenous artery to the profunda femoris, which were then averaged. Order 6 length measurements were not taken.

	Order 1	Averages	Order 2	Averages	Order 3	Averages	Order 4	Averages
	Length	Diameter	Length	Diameter	Length	Diameter	Length	Diameter
ALI	86.24144	10.48882	98.03744	11.15917	96.1163	17.31361	140.5446	23.19053
ARNI	98.48015	9.264024	108.304	11.14781	133.4424	16.31226	164.4111	24.63949
BLI	105.3227	5.881529	92.59553	7.14922	88.40336	12.03754	159.3638	19.80035
BRNI	104.6846	7.538469	110.6882	11.31782	127.2148	14.46092	164.8611	23.61522
CLI~	*	*	*	*	*	*	*	*
CRNI~	*	*	*	*	*	*	*	*
DLI	103.3425	7.515439	100.2453	9.759459	115.5508	14.03512	147.8439	20.46152
DRNI	84.8262	6.605741	88.46701	9.134799	108.9965	13.26662	136.9234	23.03898
ELI	94.34229	9.033857	123.9608	11.73478	160.0163	19.71209	122.7262	27.39432
ERNI	81.5188	8.367266	94.50524	11.17305	133.164	16.95995	157.6848	26.06367
FLI	113.9675	7.871321	107.8098	10.16107	115.7072	13.68457	154.324	21.32366
FRNI	81.7196	7.412631	76.82042	9.880871	100.207	15.058	129.9762	22.41798
GLI	83.22666	7.488039	91.13299	10.8569	103.86	13.10716	130.368	21.76098
GRNI	80.03319	7.161799	74.06476	9.130441	103.8021	13.06518	122.7058	20.59411
HLI~	*	*	*	*	*	*	*	*
HRNI~	*	*	*	*	*	*	*	*
ACLNI~	*	*	*	*	*	*	*	*
ACRNI~	*	*	*	*	*	*	*	*
BCLNI	74.40352	7.820062	82.73116	9.016035	113.8887	14.34068	143.2749	18.2212
BCRNI	70.65889	7.236068	78.79303	9.179811	139.0158	14.70697	152.7827	20.97495
CCLNI~	86.87762	9.621216	95.80622	14.15776	151.0991	18.40343	174.283	18.3284
CCRNI~	87.13613	9.684577	102.6852	13.45771	132.131	18.64002	184.8781	23.70671
DCLNI	83.80956	6.740432	89.74954	8.005522	104.2323	12.19378	147.9873	20.21939
DCRNI	81.22603	6.697802	74.09918	9.347648	81.2258	14.08127	128.9058	22.36239
ECLNI	69.05539	6.773881	77.11985	8.423853	109.9014	13.04511	144.1363	23.69298
ECLNI-Auto	59.20069	7.302052	67.49971	8.122164	96.84117	11.44632	124.4901	21.13834
ECRNI	69.1419	6.718039	83.01088	8.136526	92.96706	13.07546	134.3272	19.23071
ECRNI-Auto	60.6356	6.101981	67.27136	8.350832	75.22914	11.97415	114.5164	15.0488
FCLNI	75.49206	6.307358	84.78467	7.52497	94.99223	11.19354	120.4318	18.52443
FCLNI-Auto	69.95467	6.944222	75.57411	7.377219	80.24982	10.76628	97.02094	16.79549
FCRNI	76.87815	6.821504	78.77254	9.554703	88.404	13.31857	103.0412	19.17607
FCRNI-Auto	67.41219	6.910965	65.40293	8.506379	70.39465	11.78595	77.18841	19.28985
GCLNI	77.01696	7.072759	83.37722	9.182634	101.2262	14.63539	138.3154	21.6289
GCRNI	84.89486	7.834427	90.78625	10.14882	102.2305	15.65571	127.1546	20.08568
HCLNI	95.01164	7.815466	103.8533	11.84432	159.8453	17.76566	221.1134	25.06318
HCRNI~	89.49373	8.459142	106.5954	11.40382	132.2133	17.92901	201.8502	24.5897

	Order 5	Averages	SA	2	3	4	MB	Average
	Length	Diameter						
ALI	142.4357	33.16433	66.087	56.473	53.993	43.494	45.217	53.0528
ARNI	260.8131	30.3156	32.125	31.304	36.425	35.347	39.903	35.0208
BLI	194.4492	27.78067	45.625	40.87	42.688	41.739	33.958	40.976
BRNI	289.7884	27.13387	43.283	34.036	29.89	54.517	35.205	39.3862
CLI~	*	*	41.739	56.279	45.782	35.604	24.348	40.7504
CRNI~	*	*	24.348	34.9	33.011	27.826	25.169	29.0508
DLI	302.904	26.05036	55.189	45.515	48.666	40.821	27.254	43.489
DRNI	212.1664	28.42638	33.768	38.132	38.261	24.348	32.125	33.3268
ELI	274.4226	33.8706	65.579	64.123	68.639	54.437	51.972	60.95
ERNI	191.0467	31.07833	38.695	33.865	29.565	32.125	31.207	33.0914
FLI	204.4563	30.16407	55.671	51.507	63.234	45.472	46.077	52.3922
FRNI	315.4578	23.293	26.908	33.986	30.419	33.189	28.929	30.6862
GLI	181.1062	27.996	29.855	30	29	20.963	17.236	25.4108
GRNI	155.7559	26.01423	29.153	31.497	41.017	31.891	25.589	31.8294
HLI~	*	*	36.24	46.435	65.761	91.734	48.433	57.7206
HRNI~	*	*	*	*	*	*	*	
ACLNI~	*	*	18.282	16.726	24.051	17.586	12.205	17.77
ACRNI~	*	*	28.518	24.375	16.397	19.744	22.029	22.2126
BCLNI	117.4022	24.96456	34.956	22.392	20.95	24.334	18.332	24.1928
BCRNI	117.3414	26.21171	36.314	17.413	20.888	23.623	20	23.6476
CCLNI~	*	*	24.928	22.616	24.021	24.458	20.323	23.2692
CCRNI~	*	*	34.281	28.881	19.829	17.401	19.964	24.0712
DCLNI	167.1221	31.43587	30.022	32.977	35.984	29.571	27.419	31.1946
DCRNI	132.6518	29.8144	40.833	30.744	31.413	29.769	24.595	31.4708
ECLNI	168.3624	28.93325	30.373	30.417	31.905	30.774	30.593	30.8124
ECLNI-Auto	110.2174	28.58698	x	x	x	x	x	
ECRNI	168.5718	23.083	31.926	26.971	32.361	21.809	32.361	29.0856
ECRNI-Auto	138.5507	20.14493	x	x	x	x	x	
FCLNI	143.8183	26.88875	29.527	28.164	25.217	23.494	27.635	26.8074
FCLNI-Auto	121.7391	25.86958	x	x	x	x	x	
FCRNI	148.5342	26.84631	27.467	33.226	33.089	32.484	29.331	31.1194
FCRNI-Auto	131.7726	23.41137	x	x	x	x	x	
GCLNI	120.9407	24.41	42.064	38.507	28.748	30.879	30.285	34.0966
GCRNI	136.598	33.20075	55.223	43.695	32.268	38.261	31.316	40.1526
HCLNI	193.6465	18.6795	41.339	34.826	31.22	33.055	22.575	32.603
HCRNI~	*	*	27.826	16.901	27.32	26.292	27.253	25.1184

Appendix H – Total Vessel Counts

	Order 1	Order 2	Order 3	Order 4	Order 5
ALI	280	195	76	17	3
ARNI	288	148	74	43	10
BLI	376	173	74	23	6
BRNI	292	201	90	36	15
CLI~	*	*	*	*	*
CRNI~	*	*	*	*	*
DLI	581	292	146	69	14
DRNI	499	289	133	58	8
ELI	286	159	67	19	5
ERNI	623	317	125	49	3
FLI	549	303	129	56	15
FRNI	567	341	146	52	6
GLI	487	268	114	44	18
GRNI	783	417	197	75	13
HLI~	*	*	*	*	*
HRNI~	*	*	*	*	*
ACLNI~	*	*	*	*	*
ACRNI~	*	*	*	*	*
BCLNI	698	318	135	35	9
BCRNI	575	280	116	38	7
CCLNI~	201	113	42	5	*
CCRNI~	241	119	52	7	*
DCLNI	711	337	147	54	15
DCRNI	516	287	104	51	5
ECLNI	755	373	147	55	8
ECLNI-Aut	x	x	x	x	x
ECRNI	695	348	148	49	6
ECRNI-Aut	x	x	x	x	x
FCLNI	777	401	181	54	8
FCLNI-Aut	x	x	x	x	x
FCRNI	916	464	195	60	13
FCRNI-Aut	x	x	x	x	x
GCLNI	439	224	83	31	6
GCRNI	485	232	95	28	8
HCLNI	354	191	79	22	2
HCRNI~	353	191	67	10	*
Average	544.8696	285.1304	121.7826	44.26087	8.826087

Appendix I – JMP output of Tukey-Kramer results for sham versus control and sham versus ligated

Level	Least Sq Mean
Sham,5 A	237.50472
Sham,4 B	146.09374
Control,5 B C	140.73944
Control,4 B C D	129.24232
Sham,3 B C D E	117.80446
Control,3 B C D E	100.76863
Sham,2 C D E	92.14160
Sham,1 D E	88.54376
Control,2 D E	81.09237
Control,1 E	76.55996

Levels not connected by same letter are significantly different.

Level	Least Sq Mean
Sham,6 A	33.198800
Control,6 A B	28.359775
Sham,5 B	27.338944
Control,5 B	26.362135
Sham,4 B C	23.023618
Control,4 C D	18.896860
Sham,3 D E	14.482530
Control,3 E F	12.698495
Sham,2 F G	9.926175
Control,2 G	7.804402
Sham,1 F G	7.353698
Control,1 G	5.592468

Levels not connected by same letter are significantly different.

Sham versus control - Length

Level	Least Sq Mean
Sham,5 A	237.50472
Ligated,5 A	216.62899
Sham,4 B	146.09374
Ligated,4 B C	142.52843
Sham,3 B C	117.80446
Ligated,3 B C	113.27565
Ligated,2 B C	102.29698
Ligated,1 B C	97.74053
Sham,2 C	92.14160
Sham,1 C	88.54376

Levels not connected by same letter are significantly different.

Sham versus control - Diameter

Level	Least Sq Mean
Ligated,6 A	46.842725
Sham,6 B	34.752782
Ligated,5 B C	30.662945
Sham,5 B C D	28.545200
Sham,4 C D	24.229875
Ligated,4 D E	23.147167
Ligated,3 F H	15.806955
Sham,3 E F G	15.688787
Sham,2 F G H I	11.132431
Ligated,2 F G H I	10.962042
Ligated,1 G I	8.871776
Sham,1 H I	8.559955

Levels not connected by same letter are significantly different.

Sham versus ligated - Length

Sham versus ligated - Diameter

Appendix J – Statistical Setup in JMP for sham versus control and sham versus ligated

The screen shots on the following page show how to set up a repeated measures analysis in JMP. In the JMP home window access this screen by going to Analyze>Fit Model. In the screen shots, behind the fit model window shows how the data was organized. The Y variable is what is being measured; for this analysis it was either vessel length or diameter. The construct model effects window is where the different treatments, experimental units and effects are placed for the correct analysis. A) The model fit for the sham versus control length analysis. The treatment (sham or control) is nested within the mouse variable because treatments were delivered across different mice. B) The model fit for the sham versus ligated length analysis. Blocking on the mouse was done to remove variability within the experiment because the treatment (sham or ligation) was done on the same mouse. This is why the added “Mouse” variable is seen in the construct model effects window as well as mouse being crossed with treatment instead of being nested as before.

	Mouse	Treatment	Order	Length	Diameter
1	ARNI	Sham	1	98.48015278	9.264024306
2	ARNI	Sham	2	108.3039932	11.14781081
3	ARNI	Sham	3	133.4423784	16.31225676
4	ARNI	Sham	4	164.4110698	24.63948837
5	ARNI	Sham	5	260.8131	30.3156
6	ARNI	Sham	6		35.0208

Report: Fit Model - JMP

Model Specification

Select Columns: Mouse, Treatment, Order, Length, Diameter

Pick Role Variables:

- Y: Length (optional)
- Weight: optional numeric
- Freq: optional numeric
- By: optional

Construct Model Effects:

- Treatment
- Mouse[Treatment]& Random
- Order
- Treatment*Order

Degree: 2

Attributes: Random Effect, Response Surface Effect, LogVariance Effect, Mixture Effect

Personality: Standard Least Squares

Emphasis: Minimal Report

Method: REML (Recommended)

☒ Unbounded Variance Components

☐ Estimate Only Variance Components

Buttons: Help, Run, Recall, Remove

Keep dialog open: ☐

	Mouse	Leg	Treatment	Order	Length	Diameter
1	A	R	Sham	1	98.48015278	9.264024306
2	A	R	Sham	2	108.3039932	11.14781081
3	A	R	Sham	3	133.4423784	16.31225676
4	A	R	Sham	4	164.4110698	24.63948837
5	A	R	Sham	5	260.8131	30.3156
6	A	R	Sham	6		35.0208

Report: Fit Model - JMP

Model Specification

Select Columns: Mouse, Leg, Treatment, Order, Length, Diameter

Pick Role Variables:

- Y: Length (optional)
- Weight: optional numeric
- Freq: optional numeric
- By: optional

Construct Model Effects:

- Mouse
- Treatment
- Mouse*Treatment& Random
- Order
- Treatment*Order

Degree: 2

Attributes: No Intercept

Personality: Standard Least Squares

Emphasis: Minimal Report

Method: REML (Recommended)

☒ Unbounded Variance Components

☐ Estimate Only Variance Components

Buttons: Help, Run, Recall, Remove

Keep dialog open: ☐

Appendix K – Femoral Artery Ligation Checklist

Date _____	Hindlimb Ischemia Surgery - Ligation	Initials _____
Mouse Information	_____35. Spray right hindlimb with Nolvasan	
DOB: _____	_____36. Return animal to anesthesia box	
Sex: _____	_____37. Apply 4x4 gauze sponge to heat pad to protect animal from excessive heat	
Tag: _____	_____38. Affix non-rebreathing circuit to surgery table w/ chemistry clamp	
Genotype/strain: _____	_____39. Lay animal supine on circulating heat pad w/ nose in nose-cone	
Cage: _____	_____40. Insert rectal probe and set thermo-controller to 37°C	
Materials	_____41. Apply veterinary ointment to eyes to avoid drying during procedure	
Sterilize- autoclave or flash autoclave	_____42. Apply veterinary ointment to anus and place rectal probe ~1 cm into anus to monitor core-body temperature	
_____1. Standard pattern forceps (1)		
_____2. S&T forceps (2)	Surgery	
_____3. 5-45 forceps (1)	_____43. Make a small incision on the middle, medial aspect of the left thigh	
_____4. Iris scissors (1)	_____44. Extend the incision up to the abdominal wall	
_____5. Microdissection scissors (1)	_____45. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure	
_____6. Castroviejo (1)	_____46. Use cautery to remove fat pad overlying femoral a-v pair & cauterize epigastric av-pair	
_____7. Retractor (1)	_____47. Blunt dissect the femoral artery from the neurovascular bundle just downstream from the deep femoral branch	
Pre-sterilize in autoclave	_____48. Tie off the femoral artery & vein with 6.0 silk suture, just downstream to the deep femoral branch	
_____8. cotton gauze (2)	_____49. Use 6.0 polypropylene suture to close the skin	
_____9. cotton swabs (12)	_____50. Make a small incision on the middle, medial aspect of the right thigh	
_____10. 6.0 silk suture (2 x 1-inch)	_____51. Extend the incision up to the abdominal wall	
Obtained in surgery suite	_____52. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure	
_____11. sterile Petri dish w/ sterile saline	_____53. Use 6.0 polypropylene suture to close the skin	
_____12. sterile gloves	Post-Surgical	
_____13. sterile 7.0 prolene suture	_____54. Place the animal in the recovery bin, on a blue bench cover, above a heat pad and allow to recover	
_____14. heat-cautery	_____55. Turn flow meter down to 0, turn off isoflurane, and close the oxygen cylinder	
_____15. FST heat pad w/ rectal probe	_____56. Indicate surgery on cage card	
_____16. heat pad		
_____17. recovery bin & weigh boat	Notes	
_____18. depilatory cream	_____	
_____19. non-sterile cotton swabs	_____	
_____20. non-sterile cotton gauze	_____	
_____21. isolation mask & cap	_____	
_____22. analgesic (Buprenorphine)	_____	
Surgery preparation		
_____23. Spray surgery area with Nolvasan		
_____24. Weigh animal in weight boat		
_____25. Place animal in anesthesia box		
_____26. Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 l·min ⁻¹		
_____27. Anesthetize animal w/ 5% isoflurane		
_____28. Give the animal an subcutaneous injection of buprenorphine (0.075mg/kg)		
_____29. Affix non-rebreathing circuit to bench-top with tape		
_____30. Reduce flow rate to 0.5-1.0 l·min ⁻¹ and the isoflurane to 1-3%		
_____31. Apply ear tag high on left ear		
_____32. Lay animal supine with nose in nose-cone		
_____33. Shave hair on the right hindlimb & lower abdomen with clippers		
_____34. Remove excess hair with depilatory cream		

Appendix L – Manual Measurement Protocol

- 1) Calibrate ImageJ (see figure A below)
- 2) Select the freehand lines tool (see figure B below) to trace the vessel segments that need measuring.
- 3) Press <Ctrl+M> to measure the segment length. Note: Vessel length and diameter need to be measured separately, and it's easiest to measure a vessels length, followed by the diameter, as opposed to measuring all the lengths and then all the diameters, which makes it difficult to organize in the results window. Measurements can then be copied into Excel to be analyzed.

Steps to measure an image manually in ImageJ. A) How to set the proper scale. B) How to select the freehand line for tracing a vessel.

Appendix M – Photoshop CS3 Photomerge Protocol

First, open all the files to photomerge, select the photomerge function from the automate menu. Next, select all the files to be photomerged. Photomerge will attempt to automatically arrange all the images into a single panorama. Because of the intricacies of the vessels, photomerge sometimes fails at automatically arranging the images. In this instance, manual arrangement is necessary. As a check I would name my image files in order to make sure Photoshop arranged them properly and for ease of manual arrangement.

Appendix N – T-test statistical results for vessel count

	Order 5		Order 4		Order 3		Order 2		Order 1	
	Ligated	Sham	Ligated	Sham	Ligated	Sham	Ligated	Sham	Ligated	Sham
A	3	10	17	43	76	74	195	148	280	288
B	6	15	23	36	74	90	173	201	376	292
D	14	8	69	58	146	133	292	289	581	499
E	5	3	19	49	67	125	159	317	286	623
F	15	6	56	52	129	146	303	341	549	567
G	18	13	44	75	114	197	268	417	487	783
Mean	10.16666667	9.166667	38	52.16667	101	127.5	231.6666667	285.5	426.5	508.6667
Std Error	2.54842	1.81506	8.84685	5.51009	13.52528	17.77404	25.89423	39.75907	53.6313	79.04963
	p = 0.75583		p = 0.20393		p = 0.26286		p = 0.28302		p = 0.40985	

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