

Development of a Blood Flow Measurement Protocol Using Particle Image Velocimetry

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PROJECT INFORMATION

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ABSTRACT

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Peripheral Arterial Occlusive Disease (PAOD) is an acquired inflammatory disease where a peripheral artery becomes occluded due to the buildup of atherosclerotic plaques. In patients that possess collateral arteries, an occlusion can lead to shear induced outward remodeling, arteriogenesis, of these collaterals, partially restoring blood flow. However, newly remodeled collaterals exhibit reduced functional vasodilation, which may impair normal activity, such as ambulation. To model chronic ischemia and arteriogenesis in collaterals, a femoral artery ligation in a murine hindlimb is commonly performed. Previous efforts by our group involved measurements of collateral artery diameter to assess the impact of arteriogenesis on functional vasodilation/vascular reactivity; however diameter measurements are not as descriptive as an assessment of flow, and performing particle image velocimetry allows the change in blood flow control to be investigated. Particle image velocimetry was performed in the profunda femoris artery of unoperated murine hindlimbs with 3 μ m fluorescent microspheres. Resting and vasodilation measurements were assessed for protocol validation. As expected, muscle stimulation increased flow significantly compared to that assessed at resting, accordingly, all other measured parameters also increased significantly.

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“Life is not measured by the number of breaths we take, but by the moments that take our breath away.”

-Unknown

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Chapter 1: INTRODUCTION

Atherogenesis

Atherogenesis is the buildup of atherosclerotic plaques along the walls of an artery [1] (Figure 1). Turbulent shear, at artery branch points, along with concurrent factors, place an individual at an increased risk of this disease.

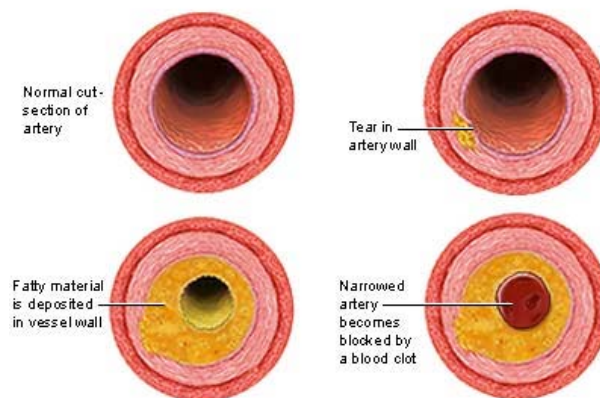


Figure 1: Progression of Atherosclerosis. Progression of atherosclerosis, which eventually leads to occlusion, in a blood vessel [2].

Endothelial cells which line the luminal surface of an artery become activated by turbulent shear, causing them to contract from one another, display adhesion proteins on their surface, and secrete chemokines that attract passing monocytes. Through integrin/adhesion protein binding, monocytes bind to the endothelial cell and eventually extravasate into the subendothelial space, becoming macrophages. Low density lipoproteins (LDLs) also extravasate from the blood stream into the subendothelial space where they react with superoxide released by macrophages and become oxidized LDL. Oxidized LDL further activates the endothelium and is also recognized by macrophages and endocytosed. As a result, macrophages filled with oxidized LDL become lipid-laden foam cells.

When a macrophage endocytoses oxidized LDL, antigen processing and presentation occur. T-helper cells recognize the oxidized LDL antigen presented on the surface of the

macrophage and release interferon gamma ($\text{INF } \gamma$), $\text{INF } \gamma$ further activates the endothelial cells and macrophages. Activated macrophages release pro-inflammatory cytokines such as tumor necrosis factor alpha ($\text{TNF-}\alpha$), mucosinoids, proteases, and coagulation factors. $\text{TNF-}\alpha$ leads to the proliferation and migration of smooth muscle cells (SMCs), and further activates the endothelium; mucosinoids induce vasospasm in SMCs, proteases disrupt forming plaques by rupturing the endothelial layer, and coagulation factors released into the blood stream cause thrombosis.

All the aforementioned factors contribute to the build-up of atherosclerotic plaques in the subendothelial space and the occlusion of an artery. Unfortunately, if nothing impedes the atherogenic process, a condition that can be likened to that of a positive feedback loop, once the disease begins it will progress and become exacerbated.

Peripheral Arterial Occlusive Disease

Peripheral Arterial Occlusive Disease, commonly referred to as PAOD, is an acquired inflammatory disease where a peripheral artery becomes occluded due to the buildup of atherosclerotic plaques. In patients, PAOD generally manifests itself in the femoral artery which becomes progressively occluded, and blood flow to the lower region of the limb is significantly decreased [3] (**Figure 2**). In the United States, approximately 8 million individuals suffer from PAOD, including approximately 12-20% of individuals over 60 years of age [3].



Figure 2: Peripheral Arterial Occlusive Disease (PAOD). PAOD is the buildup of atherosclerotic plaques in the peripheral circulation, such as in the leg [4].

In many individuals, PAOD may be asymptomatic, however it is common for PAOD patients to experience intermittent claudication, ischemic leg pain, while walking or exercising. In extreme cases of PAOD, patients with critical limb ischemia experience pain during rest. Furthermore individuals with PAOD have an increased risk of developing cerebrovascular or coronary artery disease, which may lead to stroke or a heart attack respectively [5].

The occurrence of PAOD increases with age in both men and women and is more common in African Americans, Non-Whites, Hispanics, and Asians [6]. Risk factors of symptomatic ischemic disease include a high cholesterol diet, increased LDL in the blood stream, and influences that damage the endothelial lining such as hypertension, diabetes, and smoking.

If PAOD is left untreated and the vessel continues to occlude to the point where little or no flow is present, the tissue downstream of the occlusion can become ischemic, i.e. flow is not sufficient to support metabolic demands [7]. If ischemia worsens to the point of tissue necrosis, it is possible that the limb may be amputated; however in many cases a patient will experience cardiac failure (i.e. myocardial infarction) subsequent to the necessity of limb loss [8].

Treatment for Peripheral Arterial Disease

Depending on the severity of the disease, patients with PAOD are encouraged to exercise, stop smoking, and assume a healthier diet. However, in serious cases of PAOD, where the disease has become significantly exacerbated, lifestyle changes are insufficient and some form of pharmacological, endovascular, or surgical intervention is required. Pharmacotherapy is typically administered first since it is a less invasive treatment.

Pharmacotherapy can be used to treat PAOD by delivering pharmacological agents to target the inflammatory process and progression of atherogenesis or to prevent thrombus formation. Angiotensin converting enzyme (ACE) inhibitors are typically prescribed to patients as a means to alleviate hypertension and in turn decrease the endothelial activation that triggers atherogenesis [9]. Antiplatelet drugs, i.e. aspirin, are also prescribed to decrease the likelihood of thrombus formation that would completely occlude a narrow vessel [10].

Endovascular intervention involves insertion of a balloon catheter to the area of the blockage which is then expanded to clear the blockage (**Figure 3**). To ensure patency of the vessel post-angioplasty, a stent is typically threaded around the balloon catheter and when inflated the stent is expanded against the inside of the vessel to support the walls [11]. A surgical technique commonly used to treat PAOD is bypass grafting, where a vessel is harvested from another area of the body and grafted around the blockage, diverting flow past the blockage and resuming relatively normal blood flow.

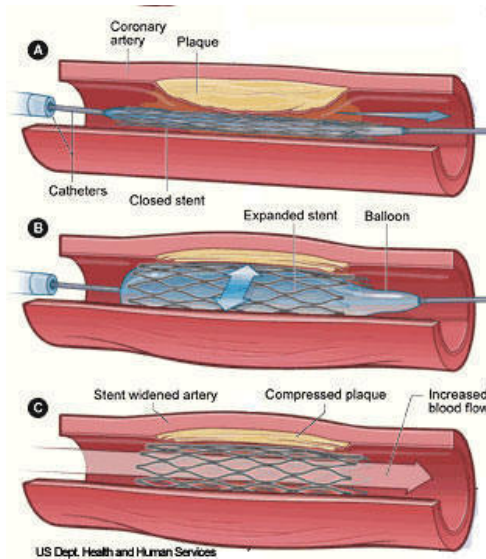


Figure 3: Endovascular Intervention. Steps involved in an endovascular intervention [12]. A – A balloon catheter with a stent threaded on the end is guided to the site of the blockage. B – Balloon is expanded, deploying the stent, keeping it in place. C – Balloon is deflated and catheter is removed, normal blood flow is restored.

In patients, a host of problems may be present in addition to PAOD, and the delivery of pharmacological agents may not be sufficient in effectively treating their condition. This necessitates the use of endovascular or surgical interventions, however endovascular treatments are not always permanent and may result in the re-occlusion of an artery [8]. Surgical intervention is typically highly invasive and may lead to multiple post-operative complications, such as prolonged healing. For these reasons promoting endogenous therapeutic revascularization is a common area of investigation.

Microcirculation and Peripheral Arterial Occlusive Disease

As mentioned previously, the peripheral arterial occlusion prevents an adequate supply of oxygenated blood from reaching the downstream vasculature, which leads to ischemia of the limb. Even if no interventions are used to treat the occlusion in a patient with PAOD, the vasculature will adapt to the modified hemodynamics, by attempting to normalize shear stresses, and these adaptations will partially restore blood supply to a given region.

When an occlusion is present in a vessel, blood will be directed through natural bypass routes, termed collateral networks. This redirection in flow increases blood flow and shear stresses through these collateral vessels, stimulating arteriogenesis, or the outward remodeling, of these collateral vessels (**Figure 4**).

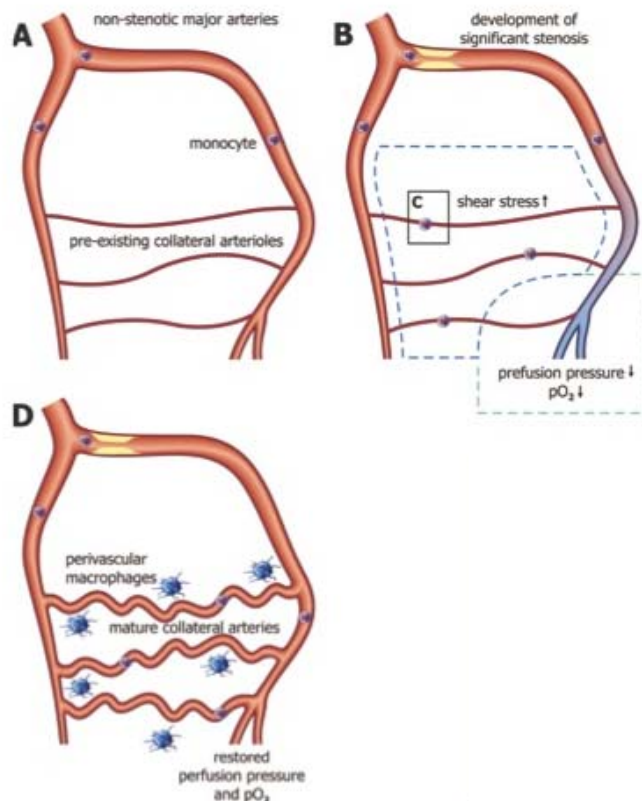


Figure 4: Arteriogenesis. The progression of arteriogenesis, outward remodeling of a collateral vessel in response to increased shear stress [13].

This increase in shear stress activates the endothelial cells (ECs) which line the luminal surface, causing them to contract from one another, display adhesion proteins on their surface, and secrete chemokines that attract passing monocytes. Through integrin/adhesion protein binding, monocytes bind to the endothelial cell and eventually extravasate into the subendothelial space becoming macrophages. These macrophages subsequently secrete matrix metalloproteinases (MMPs). Also secreted by ECs are vascular endothelial growth factor

(VEGF), and tumor necrosis factor- α (TNF- α). MMPs degrade the pre-existing matrix to allow for collateral enlargement, VEGF causes EC proliferation, and TNF- α causes smooth muscle cell (SMC) proliferation. Fibroblasts, present in the perivascular space, together with the new ECs and SMCs form a new matrix. This process occurs until the collateral vessel has enlarged to the point where shear stresses have been normalized.

Since arteriogenesis is an endogenous form of therapeutic revascularization, investigators are interested in studying this process for the purposes of a potential clinical treatment for those suffering with PAOD or other types of occlusive diseases. Chronic ischemia and arteriogenesis can be simulated by an arterial occlusion surgery and studied in a murine animal model.

Following ligation of the femoral artery in the murine hindlimb, blood flow is directed through the profunda femoris artery, through the network of vessels in the gracilis muscles, and through the saphenous artery to perfuse the hindlimb (**Figure 5**). At seven days post ligation enlargement of the profunda femoris artery is pronounced and can be studied. Currently, this remodeling is assessed by directly measuring the diameter of the profunda femoris artery in a femoral artery ligated hindlimb and comparing to that of a non-ligated hindlimb. Vascular reactivity is assessed through functional vasodilation, stimulating a muscle that is directly fed by the remodeled vessel. However, at seven days post ligation the outwardly remodeled vessel exhibits decreased vasodilation. To account for this decreased vasodilation, two differing mechanisms have been hypothesized- mechanoadaptation and endothelial cell dysfunction.

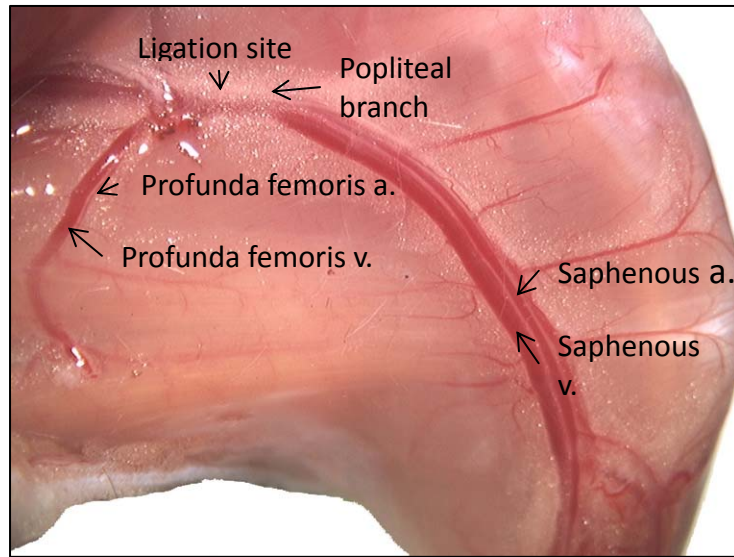


Figure 5: Collateral Network in Mouse Hindlimb. Vascular network in the mouse hindlimb.

Sources of Dysfunctional Vasoreactivity

In response to constant vasodilatory or vasoconstrictory stresses, the smooth muscle cells (SMCs) in the vascular wall will reposition themselves to normalize these stresses in a mechanism referred to as mechanoadaptation [14]. During mechanoadaptation SMCs will rearrange their attachment to other SMCs as well as other surrounding cells in the extracellular matrix (ECM) [15]. When mechanoadaptive enlargement occurs, it is hypothesized that the rearrangement of SMCs may impair their ability to relax and produce vasodilation.

Endothelial dysfunction is a second possible explanation for reduced vasoreactivity. Under normal circumstances, in response to increased shear and receptor-dependent dilators, endothelial cells (ECs) release nitric oxide (NO) which induces vasodilation of the vessel (**Figure 6**). However during inflammation, NADPH oxidase is activated by chemokines, leading to the production of superoxide which deactivates NO and thus hinders endothelial dependent vasodilation.

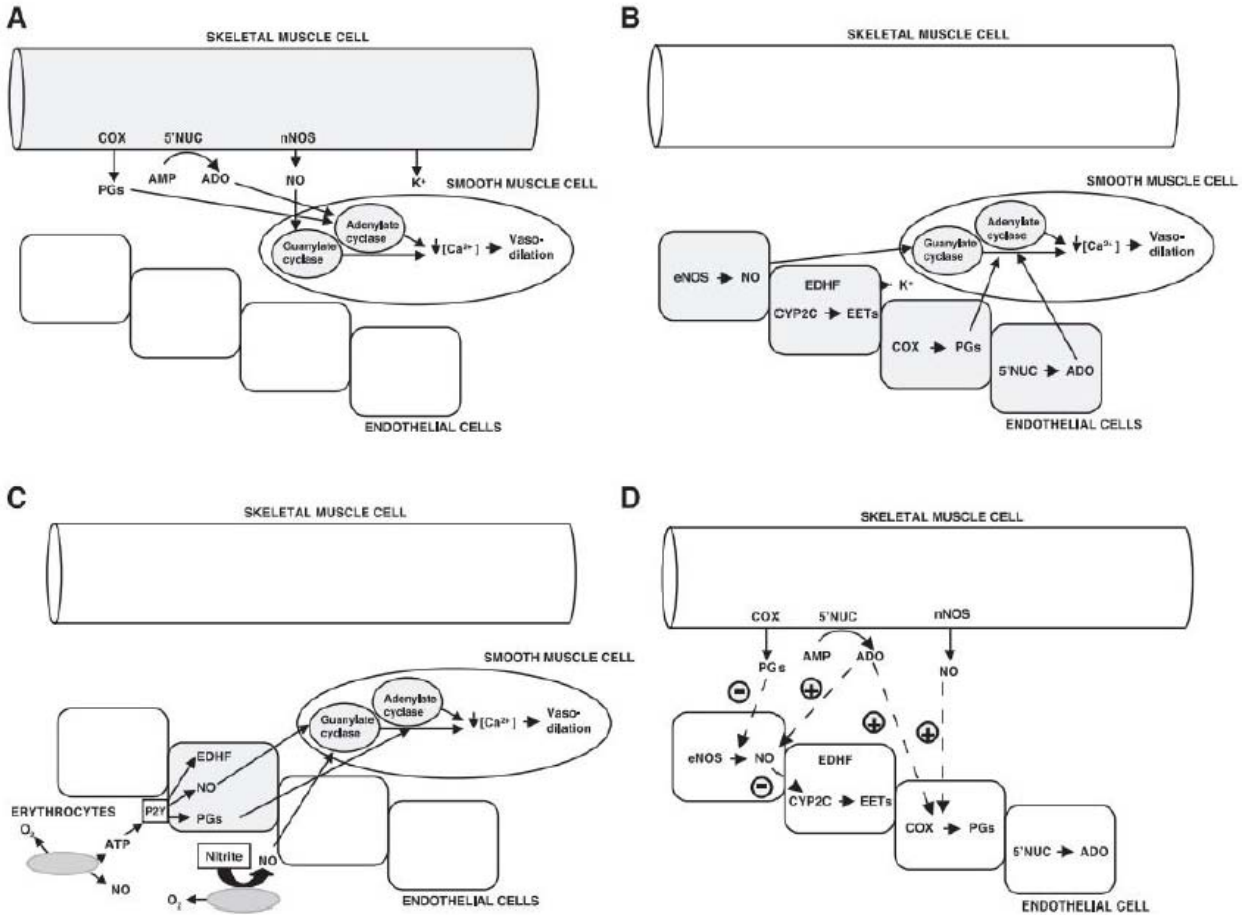


Figure 6: Normal Vasodilatory Mechanisms. Normal smooth muscle cell and endothelial cell dependent vasodilation [16].

If a patient with PAOD is capable of therapeutic revascularization, a reduced ability to vasodilate, and thus control flow, in outwardly remodeled vessels is problematic because the delivery of a sufficient amount of oxygenated blood is contingent upon proper vasoreactivity in the collateral vessel. In order to analyze flow in a vessel, diameter measurements are not adequate on their own and must be paired with velocity measurements to calculate flow. To measure velocity in a given vessel a particle image velocimetry protocol was developed.

Chapter 2: PARTICLE IMAGE VELOCIMETRY

Protocol Development

As mentioned earlier, prior to the development of this protocol, measurement of vessel diameter was the only descriptive indicator depicting vascular changes. With the implementation of particle image velocimetry another aspect of vascular remodeling can be measured, flow. Flow measurements are an important aspect of vascular remodeling because it allows for direct observation of changes in flow control, diameter measurements can only communicate so much from which the rest of the information, like changes in velocity, must be indirectly inferred.

Collateral networks consist of three zones, (**Figure 7**): the stem, midzone, and reentry, through which blood flows to re-perfuse the hindlimb. The stem region, profunda femoris artery, was selected for developing the Particle Image Velocimetry Protocol because of its superficial position; however particle image velocimetry is applicable to other regions of the mouse circulatory system.

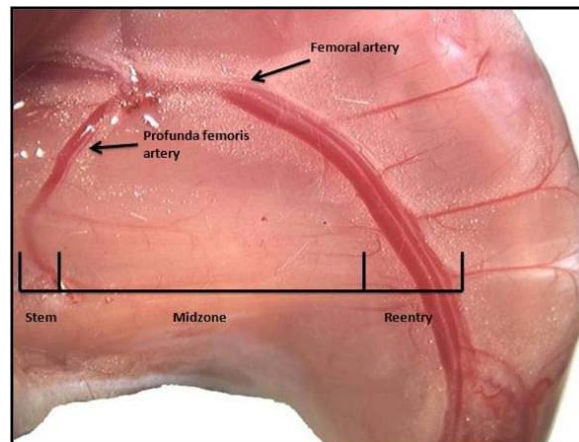


Figure 7: Zones of Collateral Network in Mouse Hindlimb. Three zones of the collateral network in the mouse hindlimb: the stem, midzone, and reentry.

Preexisting Methods

There are many methods to measure blood velocity/flow including Doppler ultrasound, Side-Stream Dark-Field Microscopy, and Fluorescence Recovery After Photobleaching (FRAP).

Below is an explanation of these approaches and reasons describing why they are not appropriate for this application of particle image velocimetry.

Doppler ultrasound is commonly used for measuring blood flow in mice, and can be used in large vessels, such as the ascending aorta of mice [17]. Doppler ultrasound involves the emission of high frequency sound waves (2-18 MHz) which are reflected from blood and excite the detector, moving fluid will result in different reflective frequencies and excite the detector differently. Doppler ultrasound is non-invasive and relatively easy to perform, however in order to obtain a unit that is small enough to use in a murine animal model it becomes increasingly expensive and thus is not as practical; additionally vessels smaller than that of a murine femoral artery may not be imaged properly. The largest caveat of Doppler ultrasound is its resolution, the ability to discern two points from one another, and in a small structure such as a murine blood vessel this drawback becomes an increasingly important concern [18]. If two points are too close together this may be detected as a single artifact by the detector and lead to an inaccurate reading; especially when blood in a vessel differs widely, depending on the location within the vessel (middle or edge) accuracy is of utmost importance.

Currently, Side-Stream Dark-Field (SDF) Microscopy is used to image the diameter of vessels as well as measure velocity in very small vessels, i.e. capillaries. SDF Microscopy implements a concentric ring of LEDs emitting a wavelength of 530 nm that is the specific to the absorbance of hemoglobin in erythrocytes. This creates a contrasting image where the vessels appear dark and the surrounding tissue appears lighter.

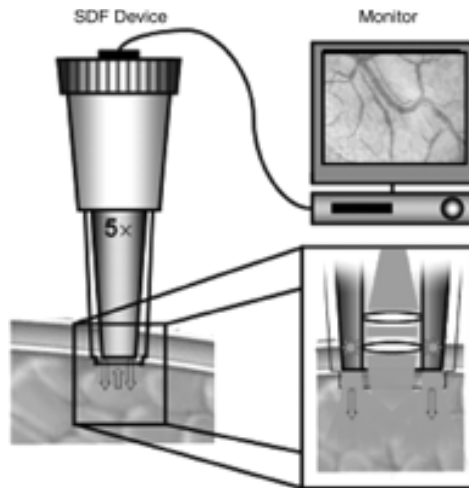


Figure 8: Side-Stream Dark-Field Microscopy. The microscope is immersed in a medium to create a column between the sample and tip, the resulting image has dark vessels and lighter surrounding tissue.

SDF microscopy is compact and relatively simple to use, the data collected is easy to obtain and interpret, and the user has fine control of the region imaged. However, to obtain velocity measurements the vessel must not be much larger than that of a capillary, $5\text{ }\mu\text{m}$; flow in arterioles can also be too fast to measure. If a vessel size exceeds that of a capillary there is not enough contiguous plasma space to provide contrast for passing erythrocytes and as a result velocity cannot be measured. Additionally, since SDF microscopy uses an immersion lens, it is easy to accidentally apply pressure on the vessel of interest and cause an artificial dilatory effect. Lastly, SDF microscopy cannot image into deep tissue; if the vessel is not superficial it is not likely to be resolved.

Another method commonly used to detect flow is fluorescence recovery after photobleaching (FRAP) which uses the inherent properties of fluorescent particles as a tracking system. FRAP makes use of photobleaching, an unavoidable property of fluorophores, that is normally considered a downside of fluorescent microscopy. To create an image using fluorescent microscopy, an excitation wavelength of light is used to excite electrons in a fluorophore and emitted wavelengths are detected by a detector. However, a fluorophore can only be excited a

finite number of times before its electrons undergo oxidation reactions and no longer fluoresce; this process is referred to as photobleaching. To perform FRAP, a region is photobleached and the time it takes for fluorescent particles to return to the area is tracked. In addition to volumetric flow, FRAP can be used to monitor the diffusive properties in a region of interest [19]. This technique is elegant and provides other benefits such as the ability to image deeper into tissue, less auto-fluorescence from the surrounding tissue, and increased resolution; all relative to widefield microscopy. However, these benefits stem from the fact that as a requirement to perform FRAP a confocal microscope which implements lasers and is one of the more expensive microscopes, must be used. Thus this method is quite complex and the intensity from the lasers can actually damage living tissue.

Taking into consideration the previously discussed methods it was evident that widefield microscopy while using particle image velocimetry (PIV) would be the best approach.

Project Rationale

In a study performed by Al-Khazraji et al., streak lengths of fluorescent red blood cells (RBCs) were measured in rat skeletal muscle arterioles [20]. RBCs were fluorescently labeled and recirculated; a fluorescent microscope, with an attached camera, positioned over desired arterioles in skeletal muscle captured passing RBCs. From these images, streak lengths of fluorescent RBCs were clearly distinguishable and measured, shutter speed of the camera allowed for velocity to be calculated by dividing the streak length by camera shutter speed. This work demonstrated that blood velocity, in small vessels, could be measured via particle image velocimetry (PIV). Instead of performing RBC labeling, this Particle Image Velocimetry (PIV) protocol utilizes circulating fluorescent microspheres of uniform size.

Project Objective: *Develop a Particle Image Velocimetry Protocol to measure blood velocity and calculate blood flow, in measured mouse skeletal muscle feed artery.*

The purpose of this objective is to establish a procedure that allows investigators to determine the impact of vascular remodeling on blood flow control.

Chapter 3: METHODS

Animal Housing and Care

Male C57Bl/6 mice between 7 and 9 weeks of age were used for all experiments according to protocols approved by the Cal Poly State University Institutional Animal Care and Use Committee. Mice were housed in the University Vivarium in micro-isolator cages within a temperature controlled room on a 12 hour light-dark cycle. Mice were monitored daily and standard chow and water were provided *ad libitum* in addition to enrichment (cage bedding, mouse house, and tunnel tube).

Mouse Preparation

Mice were anesthetized in an induction chamber using 3% vaporized isoflurane in oxygen, flowing at $0.8-1.0 \text{ l} \cdot \text{min}^{-1}$. Subsequent to initial anesthetization, mice were maintained in a plane of anesthesia using 1-2% isoflurane in oxygen flowing at $0.8-1.0 \text{ l} \cdot \text{min}^{-1}$. Mice were then weighed and moved to a preparatory bench for hair removal. Hair clippers and depilatory cream were used to remove hindlimb hair to just above the abdominal wall. Mice were transferred to a heat pad on the surgical bench where they were maintained at a core body temperature of 35°C , detected through a rectal thermistor probe for the duration of the procedure.

Microsphere Preparation

Green fluorescent microspheres, $3\mu\text{m}$ in diameter (Fischer Scientific) were prepared by vortexing for 30sec, sonication for 5min, and vortexing once more for 30sec.

Hindlimb Microdissection

An incision was made in the medial aspect of the mouse hindlimb. Blunt dissection of the surrounding connective tissue and adipose tissue was used to maximize exposure of the profunda femoris artery and vein. Perfusion of the exposed area with phosphate buffered solution (PBS) was performed periodically to prevent desiccation. The neurovascular bundle, which feeds the

epigastric fat pad, was cauterized and the entire fat pad was resected to maximize visibility. Careful blunt dissection was performed over the gracilis muscle to remove any overlying connective tissue, which minimizes the resistance to current flow and maximizes the skeletal muscle contraction.

Functional Vasodilation and Microsphere Injection

Two tungsten microelectrodes were placed on the gracilis muscle, one stimulating and one ground. To ensure correct placement of electrodes, a series of stimulating contractions were delivered with 1mA square waves, 200 μ s in duration, and a frequency of 1Hz. The area was perfused with saline and covered with plastic wrap to prevent desiccation and oxygen transport. A 30-minute equilibration period allowed for the profunda femoris artery to return to resting diameter

During the equilibration period, a skin incision was made in the neck and enlarged to expose the jugular vein. Blunt dissection of surrounding connective tissue and adipose allowed for maximum exposure of the external jugular vein, through which fluorescent microspheres were injected.

An intravital fluorescent microscope, equipped with a green filter, was positioned over the distal profunda femoris. After the equilibration period, at resting, a series of images were captured of the profunda femoris artery to measure the diameter and passing microsphere streak length; a 5x objective and Infinity software were used. Muscle stimulation was delivered for 90sec with 1mA square waves, 200 μ s in duration, at a frequency of 8Hz. Immediately following stimulus cessation a series of images were captured of the profunda femoris artery. Images were captured every minute until the diameter had returned to normal. Following the completion of the experiment, mice were euthanized via cervical dislocation.

Data and Statistical Analysis

ImageJ analysis software was used to measure vessel diameter and microsphere streak length. Microsoft Excel was used to perform statistical analysis. A $p < 0.05$ was used to denote significance from a paired T-test. The following formulas were used to calculate velocity based on streak length and flow based on velocity, with the assumption that the artery was a perfect cylinder:

Equation 1: Calculating Velocity

$$velocity [\mu\text{m/s}] = \frac{distance [\mu\text{m}]}{time [\text{s}]}$$

Equation 2: Calculating Flow Through a Cylindrical Vessel

$$flow [\mu\text{m}^3/\text{s}] = cross\ sectional\ area[\mu\text{m}^2] * velocity[\mu\text{m/s}]$$

Where:

- Distance[μm] = microsphere streak length
- Time [s] = camera shutter speed
- Area [μm^2] = calculated from the diameter of the vessel

Chapter 4: RESULTS

The motivation behind performing particle image velocimetry in the profunda femoris artery was to assess the hemodynamic changes at rest and following muscle contraction. Data obtained from functional vasodilation and intravital microscopy is presented below, microsphere streak length and vessel diameter were directly measured, from which velocity and flow were calculated. As expected, post-stimulation measurements of diameter, velocity, and flow were significantly ($p<0.05$) greater than at rest.

Diameter

As expected based on previous work, gracilis muscle contraction significantly increased profunda femoris artery diameter, resting $56 \mu\text{m} \pm 5 \mu\text{m}$ versus stimulated $86 \mu\text{m} \pm 5 \mu\text{m}$, $p<0.05$. This percent change of approximately 54% supports previous functional vasodilation findings where stimulation of the gracilis muscle leads to a functional vasodilation response in the profunda femoris.

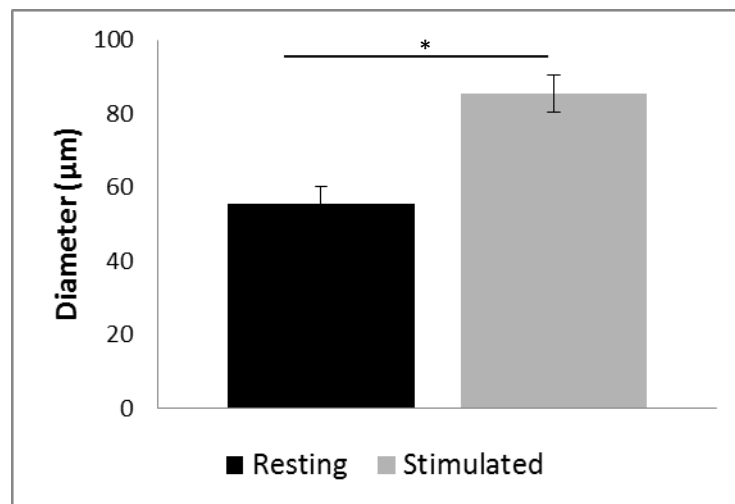


Figure 9: Profunda Femoris Diameter. Diameter of the profunda femoris artery post-stimulation was significantly larger than at rest ($p<0.05$) ($n=4$).

Blood Velocity

To calculate blood flow through the profunda femoris artery, vessel diameter and blood velocity are needed. Blood velocity was calculated using microsphere streak length and shutter speed. As expected, gracilis muscle contraction significantly increased microsphere streak length, resting $267 \mu\text{m} \pm 27 \mu\text{m}$ versus stimulated $525 \mu\text{m} \pm 53 \mu\text{m}$, $p < 0.05$; with a percent change of approximately 96%. Additionally, gracilis muscle contraction significantly increased blood velocity, resting $4814 \mu\text{m} \cdot \text{s}^{-1} \pm 1094 \mu\text{m} \cdot \text{s}^{-1}$ versus stimulated $9163 \mu\text{m} \cdot \text{s}^{-1} \pm 1732 \mu\text{m} \cdot \text{s}^{-1}$, $p < 0.05$; with a percent change of approximately 89%.



Figure 10: Visual Depiction of Post Stimulation Changes. A – Displays the profunda femoris (vessel on the left) pre stimulation and the red arrow is pointing to a microsphere and its streak length. B – Displays the profunda femoris post stimulation, as seen, the microsphere now has a longer streak length. C – Displays the profunda femoris post stimulation, as seen, there is an increased presence of microspheres in this region of interest.

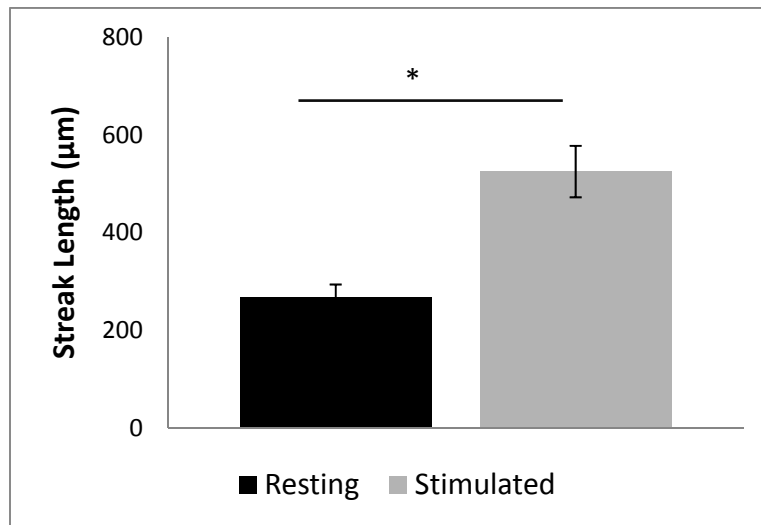


Figure 11: Streak Length of Microspheres in the Profunda Femoris. Streak length of microspheres in the profunda femoris artery post-simulation was significantly longer than at rest ($p<0.05$) ($n=5$).

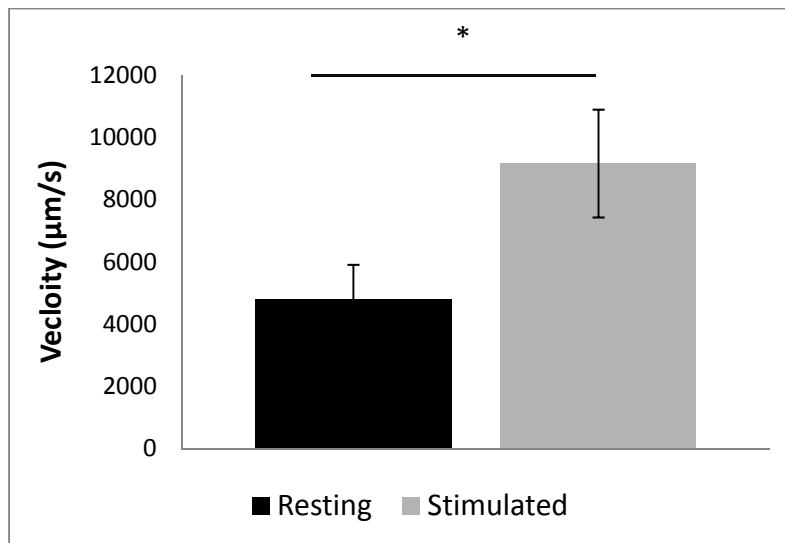


Figure 12: Blood Velocity in the Profunda Femoris. Blood velocity in the profunda femoris artery post stimulation was significantly faster than at rest ($p<0.05$) ($n=5$).

Blood Flow

Blood flow was calculated from cross sectional area and blood velocity. As expected, gracilis muscle contraction significantly increased blood flow, resting $1.3\text{E-}05 \text{ mL}\cdot\text{s}^{-1} \pm 3.9\text{E-}06$

$\text{mL}\cdot\text{s}^{-1}$ versus stimulated $5.6\text{E-}05 \text{ mL}\cdot\text{s}^{-1} \pm 1.0\text{E-}05 \text{ mL}\cdot\text{s}^{-1}$, $p<0.05$; with a percent change of approximately 96%.

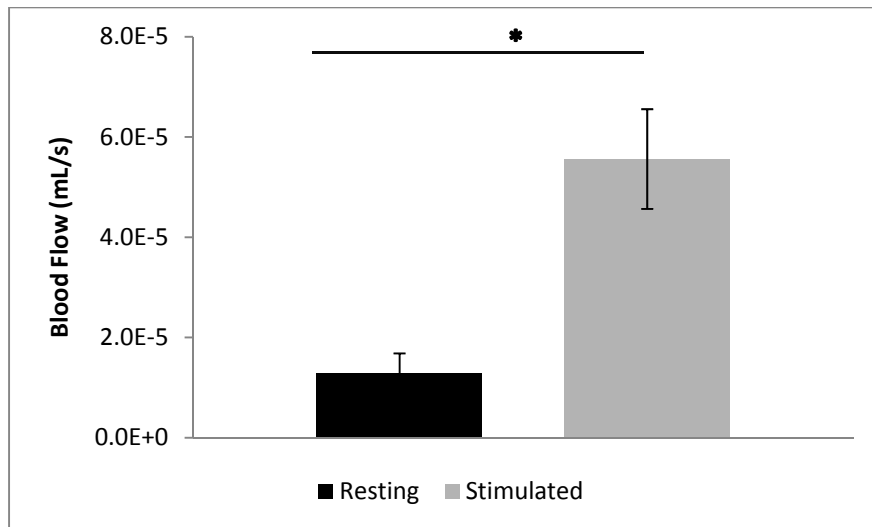


Figure 13: Blood Flow in the Profunda Femoris. Blood flow in the profunda femoris artery post stimulation was significantly faster than at rest ($p<0.05$) ($n=4$).

Chapter 5: DISCUSSION

Investigating peripheral arterial occlusive disease (PAOD) by modeling chronic ischemia via femoral artery ligation in the mouse hindlimb can be assessed by diameter measurements. However, to gain further insight into the vascular remodeling that occurs, blood flow measurements in a vessel necessary to determine the impact of vascular remodeling on blood flow control. A particle image velocimetry (PIV) protocol, utilizing circulating fluorescent microspheres, was developed as a means to calculate blood flow.

The efficacy of the blood flow protocol was determined by comparing results obtained at resting to those post stimulation of the gracilis muscle. As expected, measurements post-stimulation were significantly ($p < 0.05$) greater from those taken at rest for diameter, velocity, and flow. Functionally stimulating a given muscle simulates muscle exercise, where exercising muscle requires increased blood flow to meet metabolic demand, i.e. nutrient delivery and waste removal; thus when the gracilis muscle contracts, its feed artery, the profunda femoris artery, dilates and with this dilation comes increased blood velocity and flow, which was observed.

Limitations

The PIV protocol also has limitations. The protocol is applied assuming the profunda femoris is a perfect straight cylinder, however there is slight variability within mouse vasculature in a sense that some profunda arteries tend to curve somewhat; to account for the curved nature of the microsphere streaks, the segmented line tool is used for measurements; however this deviates slightly from the perfect cylinder assumption which may influence the calculated flows. Additionally, finding the middle “layer” of the three-dimensional vessel to capture images of passing microspheres can be somewhat challenging; however, this step is important for optimal measurements and will be more intuitive with investigator practice.

Future Work

Protocol development was performed on un-operated mice, which did not undergo any surgical modification to induce chronic ischemia. However with the establishment of the PIV protocol, this technique can now be applied to mice modeling chronic ischemia, femoral artery ligation; in combination with a murine hindlimb ischemia model, the PIV protocol allows investigators to determine how blood flow control is impacted by vascular remodeling in collateral arteries.

An additional interest of implementing the PIV protocol is using a different imaging modality, confocal or multiphoton microscopy, to assess flow in the midzone of the collateral circuit. Using confocal or multiphoton microscopy allows for imaging deeper into the tissue and eliminates an increasing amount of autofluorescence/background noise.

To determine the potential effect of a femoral artery ligation on conducted vasodilation within the hindlimb, a variation on the current functional vasodilation protocol can be performed. Instead of stimulating the gracilis muscle, the gastrocnemius (calf) muscle can be stimulated and the potential dilation and change in blood flow can be observed in the profunda femoris. This variation on the current protocol, if efficacious, adds insight into another aspect of chronic ischemia that has not been as extensively studied.

References

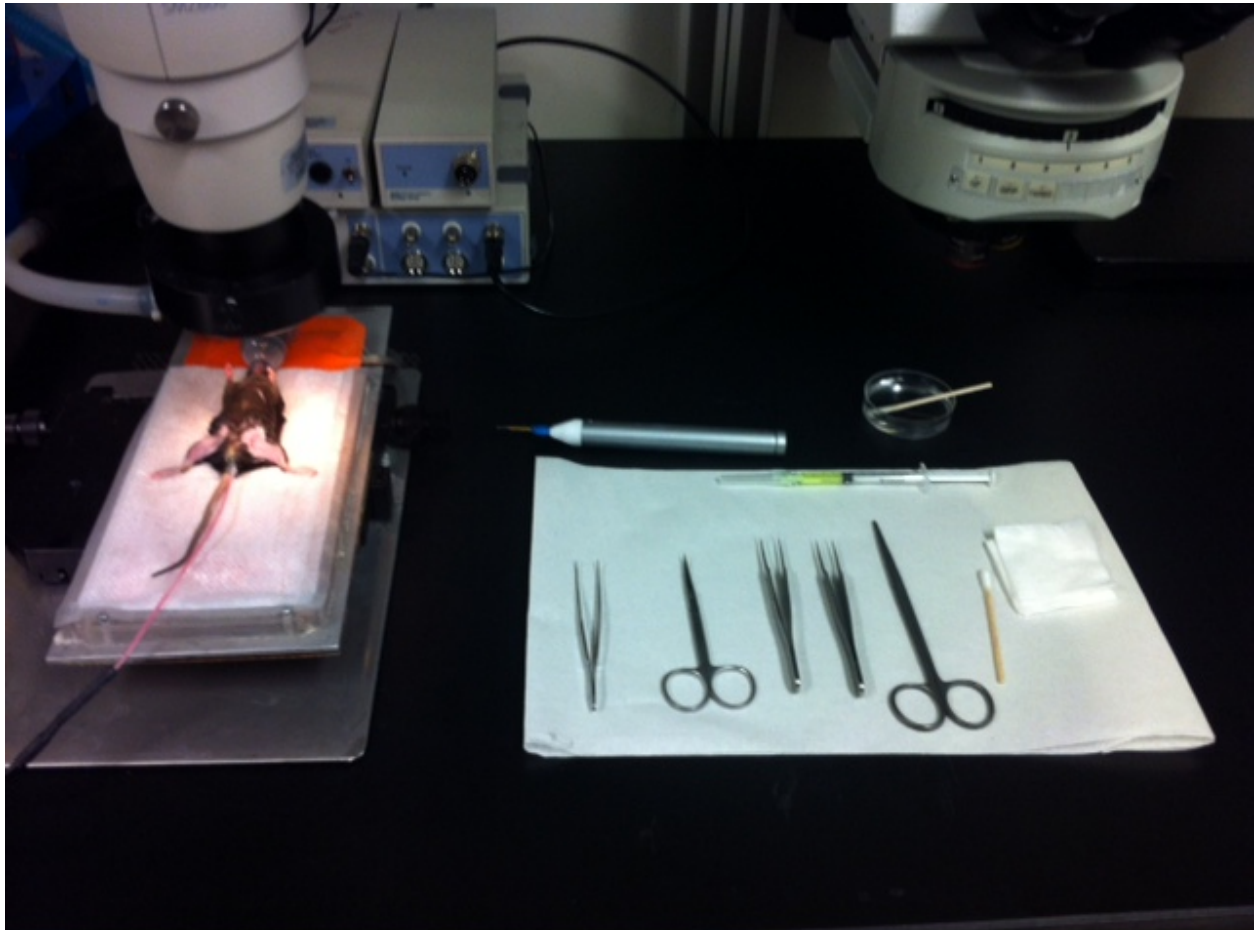
1. Libby, P., Ridker, P.M., and Maseri, A., 2002, Inflammation and Atherosclerosis, *Circulation*, v.105, p.1135-1143.
2. Dugdale, D.C., 2011, Developmental process of atherosclerosis, *MedlinePlus: Trusted Health Information for You*, <http://www.nlm.nih.gov/medlineplus/ency/imagepages/18020.htm>, (November 15, 2011)
3. Centers for Disease control and Prevention, 2011, Peripheral Arterial Disease Fact Sheet, *National Center for Chronic Disease Prevention and Health Promotion, Division for Heart Disease and Stroke Prevention*.
4. Halabi, A., 2009, The Facts About Peripheral Arterial Disease, *Michigan Medical Report*, http://www.michiganmedicalreport.com/michigan_cardiology/details/237/podcast.aspx, (November 12, 2011)
5. Hirsch, A.T., Haskal, Z.J., and Hertzner, N.R., 2006, ACC/AHA 2005 Practice Guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic), *Circulation*, v.113(11), p.e463-654.
6. Criqui, M., Vargas, V., Denenberg, J.O., Ho, E., Allison, M. Langer, R.D., Gamst, A., Bundens, W.P., and Fronek, A., 2005, Ethnicity and Peripheral Arterial Disease: The San Diego Population Study, *Circulation*, v.112, p.2703-2707.
7. Pijls, N.H.J., Bech, J.W., Gamal, M.H., Bonnier, H.J.M., Bruyne, B., Gelder, B., Michels, H.R., and Koolen, J.J., 1995, Quantification of Recrutable Coronary Collateral Blood Flow in Conscious Humans and Its Potential to Predict Future Ischemic Events, *J Am Coll Cardiol*, v.24(7), p.1522-1528.
8. Ouriel, K., 2001, Peripheral Artery Disease, *The Lancet*, v. 385, p.1257-1264.
9. Cunha, V., Tham, D.M., Martin-McNulty, B., Deng, G., Ho, J.J., Wilson, D.W., Rutledge, J.C., Vergona, R., Sullivan, M.E., and Wang, Y., 2005, Enalapril attenuates angiotensin II-induced atherosclerosis and vascular inflammation, *Atherosclerosis*, v.178, p.9-17.
10. Szczeklik, A., Kraznowski, M., Gora, P., and Radwan, J., 1992, Antiplatelet drugs and generation of thrombin in clotting blood, *Blood*, v.80, p.2006-2011.
11. Zieve, D., Chen, M.A., 2011, Angioplasty and stent placement, *Medline Plus*, <http://www.nlm.nih.gov/medlineplus/ency/article/002953.html>, (November 15, 2011)
12. McDougall, J., 2011, The Angioplasty Debacle, *The McDougall Newsletter*, <http://www.drmcDougall.com/misc/2006nl/sept/angio.html>, (November 23, 2011)

13. Schirmer, S.H., Nooijen, F.C., Piek, J.J., and Royen, N., 2009, Stimulation of collateral artery growth : travelling futher down the road to clinical application, *Heart*, v.95(3), p.191-197.
14. Martinez-Lemus, L.A., Hill, M.A., and Meininger, G.A., 2009, The plastic nature of the vascular wall: a continuum of remodeling events contributing to control of arteriolar diameter and structure, *Physiology*, v.24, p.45-47.
15. Lee, R.M.K.W, Sandow, S.L., and DeMey, J.G.R., 2009, Vascular Remodeling Arterioles: Plasticity of the Vessel Wall, *Physiology*, v.24, p.271-272.
16. Clifford, P.S., and Hellsten, Y., 2004, Vasodilatory mechanisms in contracting skeletal muscle, *J Appl Physiol*, v.97, p.393-403.
17. Hartley, C.J., Michael, L.H., and Entman, M.L., 1995, Noninvasive measurement of ascending aortic blood velocity in mice, *American Journal of Physiology: Heart and Circulatory Physiology*, v.268, p.H499-H505.
18. Coatney, R.W., 2001, Ultrasound Imaging: Principles and Applications in Rodent Research, *ILAR Journal*, v.42(3), p.233-247.
19. Olympus, 2012, Photobleaching, *Microscopy Resource Center*, <http://www.olympusmicro.com/primer/java/fluorescence/photobleaching/>, (November 12, 2011)
20. Al-Khazraji, B.K., Novielli, N.M., Goldman, D., Medeiros, P.J., and Jackson, D.N., 2012, A Simple “Streak Length Method” for Quantifying and Characterizing Red Blood Cell Velocity Profiles and Blood Flow in Rat Skeletal Muscle Arterioles, *Microcirculation*, v.19(4), p.327-335.

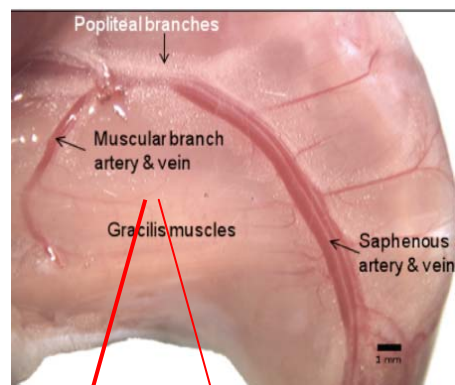
APPENDIX

Particle Image Velocimetry Setup

Instruments and equipment used for protocol development.



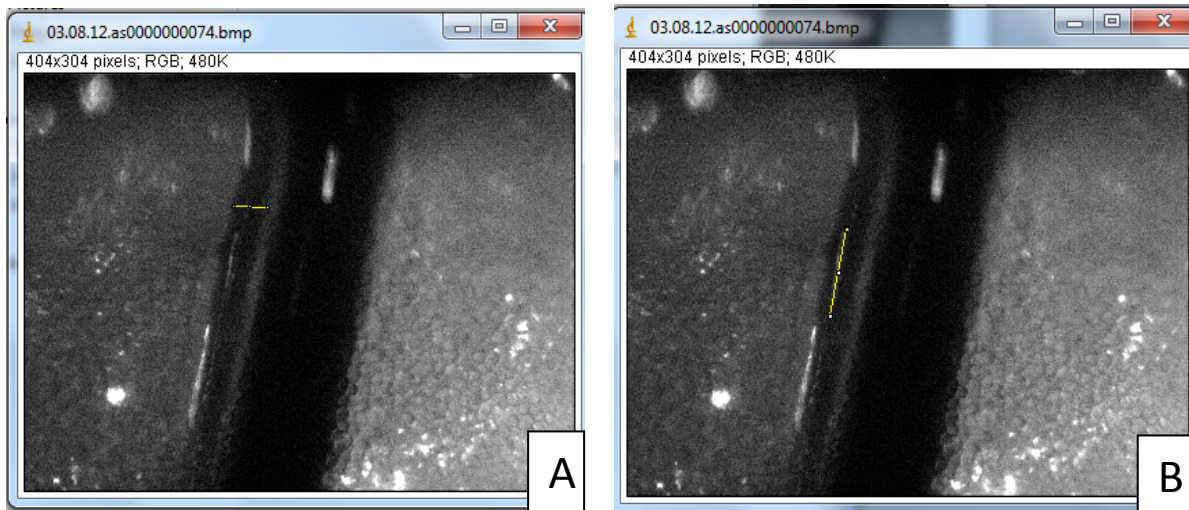
Electrode placement on the gracilis muscle for functional vasodilation of profunda femoris artery.



Imaging and Data Analysis

It should be noted that during imaging the images were binned (4x4), i.e. $\frac{1}{4}$ of the pixels in a region were used, as a means to enhance the resolution.

For image analysis the line tool in ImageJ was used to measure the diameter of the profunda femoris, from edge to edge, five measurements were made along the length of the vessel and an average of these five values were taken to obtain an average diameter. In order to measure streak length, the line tool was again used to measure from the tip of the sphere to the tail of the sphere. In order to preserve the integrity of the data, microspheres were only measured if they appeared in the middle of the vessel, since flow is most consisted in the middle of the vessel. A demonstration of measurements can be viewed below. During data analysis the size of the microsphere was accounted for by subtracting $3.1\text{ }\mu\text{m}$ from the measured streak length.



Particle Image Velocimetry Protocol

Date _____	Microsphere Injection Velocity	Initials _____
Mouse Information		
DOB: _____		
Sex: _____		
Tag: _____		
Genotype/strain: _____		
Cage: _____		
Materials		
Instruments		
____ 1.	forceps (1)	____ 32.
____ 2.	S+T forceps (2)	____ 33.
____ 3.	iris scissors (1)	____ 34.
____ 4.	microdissection scissors (1)	____ 35.
____ 5.	metzenbaum	____ 36.
Obtained in surgery suite		
____ 6.	Petri dish w/ sterile saline	____ 37.
____ 7.	Examination gloves	____ 38.
____ 8.	FST heat pad w/ rectal probe	
____ 9.	non-sterile cotton swabs	
____ 10.	non-sterile cotton gauze	
____ 11.	1 mL syringe	
____ 12.	30 gage needle	
____ 13.	3 micron fluorescent microspheres	
Surgery preparation		
____ 14.	Weigh animal	
____ 15.	Place animal in induction	
____ 16.	Open the oxygen cylinder and set anesthesia-machine flow meter to $\sim 3 \text{ l} \cdot \text{min}^{-1}$	
____ 17.	Anesthetize animal w/ 5% isoflurane	
____ 18.	Reduce flow rate to 0.5-1.0 $\text{l} \cdot \text{min}^{-1}$ and the isoflurane to 1-3%	
____ 19.	Lay animal supine with nose in nose-cone	
____ 20.	Lay animal supine on circulating heat pad w/ nose in nose-cone	
____ 21.	Insert rectal probe and set thermo-controller to 35°C	
Microsphere preparation		
____ 22.	Vortex microsphere bottle for 30 seconds	
____ 23.	Degas water bath for 5 minutes	
____ 24.	Sonicate microsphere bottle for 5 minutes	
____ 25.	Vortex microsphere bottle for 30 seconds	
____ 26.	Draw 0.2 mL of microspheres into a syringe	
Injection preparation		
____ 27.	Make an incision in the left ventral inferior neck	
____ 28.	Widen incision and blunt dissect the subcutaneous connective	
____ 29.	Blunt dissect to expose left jugular vein	
____ 30.	Inject microspheres into jugular vein	
____ 31.	Place cotton swab over injection site	
Measurement preparation		
____ 32.	Make a small incision on the middle, medial aspect of the left thigh	
____ 33.	Extend the incision up to the abdominal wall	
____ 34.	Blunt dissect the subcutaneous connective tissue to maximize surgical exposure of the profunda femoris	
____ 35.	Place wooden end of cotton swab on knee and secure to allow for isometric contraction and ensure that the hind limb is level	
____ 36.	Place negative electrode on the center of the gracilis anterior and secure electrode	
____ 37.	Place positive electrode as close as possible to the negative electrode	
____ 38.	Place mineral oil over exposed areas	
Fluorescent Microscopy		
____ 39.	Turn on mercury lamp, note time and bulb hours	
____ 40.	Open Infinity Capture.	
____ 41.	Select 404x304 (binning 4x4) Mono for Preview Resolution and Capture Resolution	
____ 42.	Select Options -> Auto-saving -> Enable Auto-Saving	
____ 43.	Set Folder, file name, start value	
____ 44.	Locate profunda femoris and adjust focus for best resolution.	
____ 45.	Note shutter speed _____	
____ 46.	Allow 30 equilibrium period to pass before taking measurement.	
____ 47.	Capture images of muscular branch and microspheres	
____ 48.	Open Lab Chart 6 open file in blood pressure measurement folder then open microscan default settings.	
____ 49.	Set stimulation frequency to 8Hz, duration to 200 μ s, and 1mA	
____ 50.	Turn on stimulator panel	
____ 51.	Stimulate muscle for 90 sec	
____ 52.	Immediately capture images and continue to capture every two minutes until vessel has returned to resting diameter	
____ 53.	Repeat steps 33-53 on the contralateral	
Analysis		
____ 54.	Open Image J	
____ 55.	For scaling if 404X304 (4x4) binning was used, 30 pixels = .1mm	
____ 56.	Record results in provided table	
Post-Surgical		
____ 57.	Cervical dislocation to euthanize animal	

Date_____

Microsphere Injection Velocity

Initials_____

Notes

Limb 1

	Diameter	Streak Length
Pre-stimulation		
Post-stimulation		

Limb 2

	Diameter	Streak Length
Pre-stimulation		
Post-stimulation		