



# **Mechanoadaptation of Feed Artery Smooth Muscle Cells in the Stem of the Collateral Circulation**

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PROJECT INFORMATION

TITLE:           Mechanoadaptation of Feed Artery Smooth Muscle Cells  
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DATE SUBMITTED:       27 September 2013

## Abstract

Many patients suffering from peripheral arterial occlusive disease (PAOD) experience intermittent claudication, pain during locomotion. Previous studies suggest that this symptom could be explained in part by impaired vasodilation in collateral arteries. In this study, femoral ligation was performed on a murine animal model, stimulating collateral outward remodeling. The mechanism by which a collateral increases its luminal diameter in response to the increase in blood flow following occlusion warrants further investigation due to impaired vasodilation following collateral remodeling. Specifically, resting diameter is elevated in the stem region of the collateral circuit, but this increase in vessel size cannot be explained by vascular smooth muscle cell (VSMC) proliferation. Therefore, we tested the hypothesis that enlargement was due to changes in VSMC overlap and/or length, a process known as mechanoadaptation. VSMC length and overlap measurements in the profunda femoris were taken from 40x confocal images. Mean VSMC length and overlap were measured 7 and 28 days post ligation. Mean length was  $222\ \mu\text{m} \pm 76$  vs  $229\ \mu\text{m} \pm 12$  in the contralateral control limb and  $310\ \mu\text{m} \pm 67$  vs  $187\ \mu\text{m} \pm 31$  in the contralateral control limb, respectively. Overlap was  $23 \pm 2$  vs  $27 \pm 2$  in the contralateral control limb and  $30 \pm 5$  vs  $30 \pm 3$  in the contralateral control limb, respectively. None of the values were significantly different. A major challenge faced included the inability of the vessel to maintain their native cylindrical shape because of the way they were flattened during storage on the microscope slides. A proposed perfusion fixation technique with 4% paraformaldehyde may allow for the excised muscle to maintain its native geometry and more VSMC overlap measurements to be taken.

**Acknowledgements:**

I would like to sincerely thank Dr. Trevor Cardinal for challenging me and pushing me to do my best. I would also like to thank the members of the Microcirculation and Tissue Repair Lab for their guidance and support. Also, thank you to all of the mice that participated in this study.

*“Yesterday is history. Tomorrow is a mystery. Today is a gift, this is why we call it the present.”*

-Bill Keane

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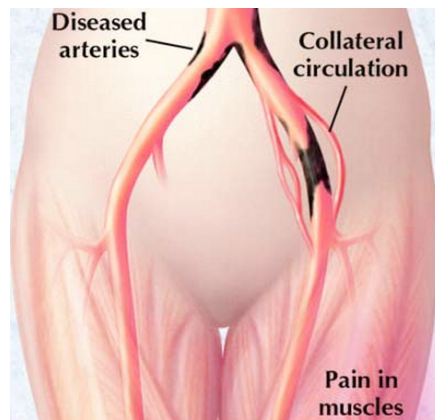




## CHAPTER 1

### Introduction to Ischemic Disease

Ischemic disease manifests as different pathologies depending on the vascular bed affected, including peripheral arterial occlusive disease (PAOD). The build up of atherosclerotic plaque results in increased blood flow and arterial wall shear stress in collateral vessels, connections that function as anastomoses and provide a natural bypass for blood around the occlusion (**Figure 1**). These collaterals can partially restore oxygen delivery and meet the metabolic demand of the downstream tissue. Patients diagnosed with PAOD experience locomotion-induced pain, referred to as intermittent claudication, which restricts their exercise tolerance and negatively affects their quality of life [6]. In the absence of well-developed collaterals, tissue downstream of an occlusion can be reperfused by stenting and angioplasty. Although these options are generally effective, they are not always a practical solution.



**Figure 1 Vascular Occlusion.** An occlusion in peripheral arteries causes a re-routing of blood, often accompanied by a structural enlargement of those vessels now supporting a larger flowrate [4].

Angioplasty includes the insertion of a balloon catheter via the femoral artery, which is deployed and inflated at the site of the narrowed lumen. The balloon pushes the

plaque against the vascular wall and flattens it. Angioplasty is not a feasible therapy for patients suffering from PAOD due to the fact that 30-50% of recipients experience a re-narrowing of the blood vessel, called restenosis, some time after the procedure [2]. Over 50% of coronary artery angioplasty recipients experienced post-angioplasty restenosis, suggesting that additional revascularization measures are necessary to decrease the probability of restenosis [11]. Another alternative treatment is stenting. A stent is a cylindrical medical device made out of woven metal wire, often Cobalt –Chromium, Stainless Steel, or Nitinol and is inserted via a femoral artery catheter. The stent is deployed at the area of occlusion, exerting a constant force against the vascular wall, which flattens the plaque. Eventually, endothelialization, the growth of endothelium over the surface of the device, establishes a new layer of cells over the stent. Stenting is sometimes not a feasible option for patients with PAOD because with the implantation of most medical devices, there poses a large risk for thrombosis [6]. Particularly with regard to drug-eluting stents, in which late-stent thrombosis is not uncommon, a lower perfusion rate was observed in patients with late-stent thrombosis [12].

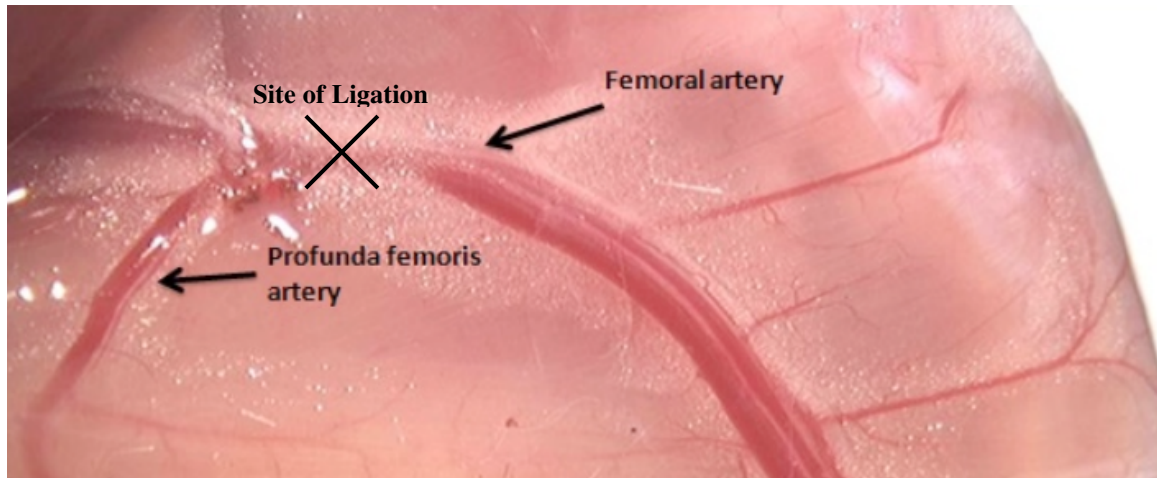
If PAOD is left untreated, and if sufficient collateral vessels are not present, hypoxic conditions may result in tissue necrosis. Limb amputation is a last resort, as it drastically reduces a patient's quality of life. Due to the issues with these two possible treatments for patients suffering from PAOD, there is a need for an alternate therapy.

Humans have collateral vessels within their circulatory system, which serve to optimize tissue perfusion. When atherosclerotic plaques reduce the ability of the vasculature to meet metabolic oxygen demands, collaterals can function as natural bypass routes. When an occlusion occurs, these collateral vessels have blood flow redirected into

them. For optimum collateral function, they must be capable of vasodilation. Unfortunately, that the enlargement processes following this redirection of blood flow into collaterals is accompanied by impaired vasodilation. Further knowledge regarding this mechanism of impairment is an essential step in the development of angiogenic therapies.

Arterial vasodilation is necessary for oxygen and nutrient delivery to tissue. This phenomenon increases a PAOD patient's risk of intermittent claudication which is a motivation behind this study. More specifically, the purpose of this study is to observe the remodeling response stimulated by collateral enlargement and its effect on vascular wall behavior.

Previous work (unpublished observations) indicates that collateral enlargement impairs vasodilation. However, because VSMC proliferation was not observed in this region of the collateral circuit (the stem), the mechanism of impaired vasodilation is unclear. Therefore, we hypothesize that smooth muscle cells reorient themselves to account for the increase in vessel diameter, called mechanoadaptation, and that the vasodilatory response is impaired during the reorientation process (**Figure 2**). Thus, further studies are needed to test this hypothesis.



**Figure 2 Gracilis Muscle.** A superficial view of a mouse's hindlimb, its femoral artery, site of ligation, and profunda femoris artery, which exhibits an increased resting diameter and impaired vasodilation at day-7 following ligation.

### Specific Aims

It is hypothesized that following femoral occlusion, the profunda femoris artery will undergo structural enlargement by mechanoadaptation, achieved by decreases in smooth muscle cell overlap (**Figure 2**). This mechanoadaptation is explored to determine its role in the impairment of vasodilation mechanisms in collateral vessels. The results from this study may explain some of the symptoms of ischemic disease. Developing a protocol for imaging the vessels and measuring VSMC length and overlap will allow for data analysis that will either support our hypothesis or not.

**Aim 1 – Protocol for imaging vessels**

This experiment will test and validate the protocol for imaging excised gracilis muscles. The current protocol involves the whole mounting of each specimen along with mounting media onto glass microscope slides. Each slide is placed under fluorescent light or a confocal laser for imaging.

**Aim 2 – Measurement of VSMC length and overlap**

Using an imaging software, cells will be randomized and measurements of their length and overlap will be taken. According to G\*Power Software, 5 cells from each profunda will need to be measured in order to have a power value of 0.98. It should be noted that although 5 is the suggested sample size, the flattened geometric nature of each muscle may not allow for this many cells to be measured [17].

## **CHAPTER 2**

### **Animal Model and Study Overview**

The animal care protocol used in this study was approved by IACUC (Institutional Animal Care and Use Committee). PAOD was modeled and collateral enlargement was induced in eight C57Bl/6 mice aged 7-9 weeks by ligating the left hindlimb femoral artery between the profunda femoris and popliteal artery-vein pairs. A sham surgery was performed on the right hindlimb of each mouse. The left hindlimb provided an animal model for PAOD and collateral enlargement while the right hindlimb served as the control. Each muscle was stained for alpha smooth muscle actin in order to view individual smooth muscle cells with fluorescent and confocal microscopy.

### **Microscopy**

Post staining protocol involved the storage of 16 gracilis muscles in PBS. During staining, the muscles were washed and stained with alpha smooth muscle actin for 3 nights at 4°C. After staining, each sample was mounted on a glass microscope slide in preparation for fluorescent and confocal imaging. For all confocal images, a 40x oil immersion objective was used with a numerical aperture of 0.75.

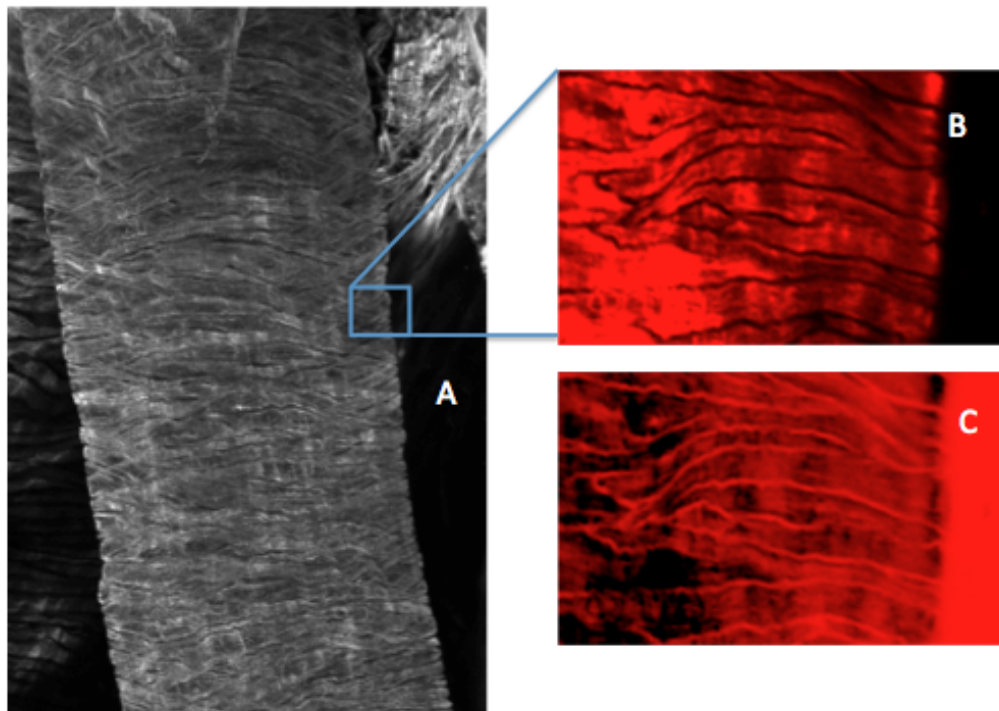
### **Morphometry**

Mean cell and overlap lengths were measured with 3 different cells on each profunda. The equations and methods used can be found in Appendix B.

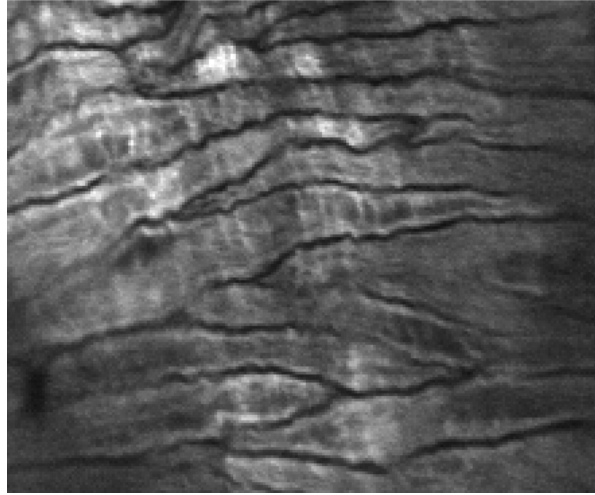
## CHAPTER 3

### Results

The goal of this study was to explore the extent which mechanoadaptation explains impaired vasodilation. Analysis of confocal images of the profunda femoris artery included measurement of cell overlap and individual cell length. Figure 3 is an example of a 40x magnified confocal image of a profunda sample.

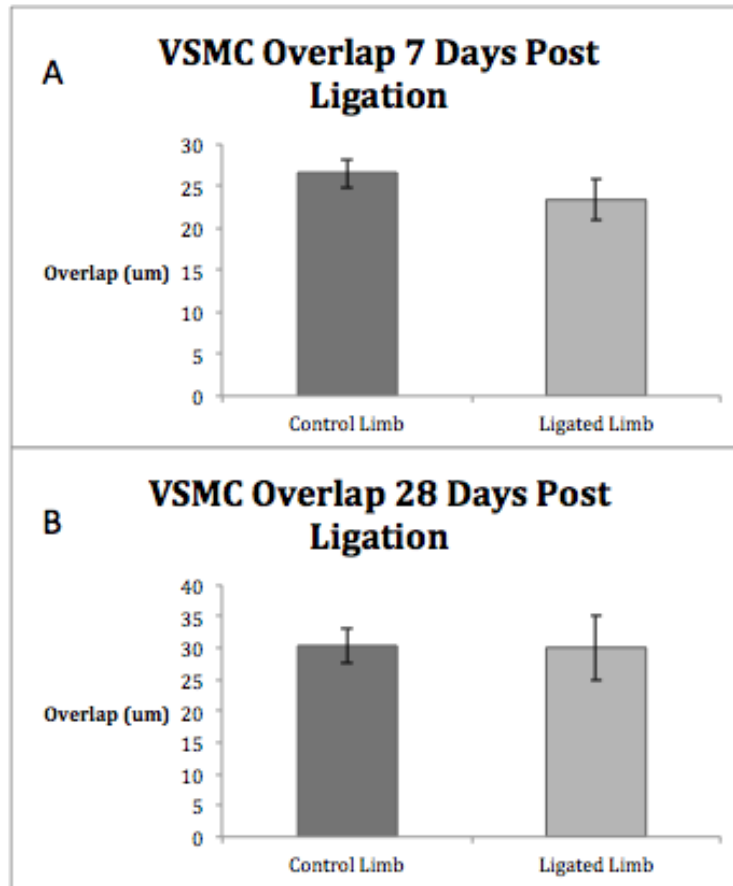


**Figure 3 Color Filter Applied to Vascular Smooth Muscle Cell Overlap.** The profunda femoris (A) and two magnified images of a wall segment with apparent cell overlap. (B) has a red filter for improved visualization and (C) has been inverted for distinction of the geometric boundaries of each cell.



**Figure 4 Smooth Muscle Cell Overlap.** This is a magnified image of multiple instances of smooth muscle cell overlap. Overlap length measurements were made using Equation 1 (**Appendix C**).





**Figure 5 Quantitative Extent of Smooth Muscle Cell Overlap.** (A) shows the overlap length as a percentage of vessel diameter 7 days post ligation. (B) shows the overlap length as a percentage of vessel diameter for mice 28 days post surgery.

Tables I and II below list the measured smooth muscle cell lengths, overlaps, and their mean standard errors for control and ligated limbs, 7 and 28 days post ligation.

**Table I: VSMC Length Data**

Limb/Day	Cell Length ( $\mu\text{m}$ )	$\pm$ MSE
Control/7	229	12
Control/28	187	31
Ligated/7	222	76
Ligated/28	310	67

**Table II: VSMC Overlap Data**

Limb/Day	Overlap Length ( $\mu\text{m}$ )	$\pm$ MSE
Control/7	27	2
Control/28	30	3
Ligated/7	23	2
Ligated/28	30	5

## CHAPTER 4

### Discussion

PAOD is a serious condition in which blood flow downstream of occluded vessels is diminished. When a patient has collateral vessels, symptoms may be improved. However, if these collaterals are not dilating properly, sufficient nutrient delivery to tissues during increased metabolic demand may not be met. Moreover, intermittent claudication, hypoxic pain with exercise or locomotion, decreases quality of life and necessitates further investigation into the mechanism underlying impaired vasodilation. Collateral enlargement was stimulated in a murine animal model to examine its impact on vasodilatory ability. It was hypothesized that rearrangement of smooth muscle cells would occur around the vessel's circumference to change luminal diameter. This mechanoadaptation could be a stimulus that contributes to the impairment of vasodilation. It is apparent that one way that a collateral responds to changes in blood flow is increasing or decreasing overlap between two or more individual cells. This phenomenon is a positive change initially because metabolic demands of downstream tissues may be met. However, when further vasodilation is necessary, the vessel is not able to accommodate.

Rearrangement, consistent with our hypothesis, would result in a decrease in the right hindlimb 7 days post ligation, and a return to control length 28 days post ligation. Although this rearrangement was observed as well as a general trend, not enough measurements were made due to poor muscle storage. A maximum of 4 measurements were able to be made on each profunda but G\*Power Analysis Statistical Software suggests that 5 should be measured to increase the power of the experiment [17]. At 40x magnification, each artery had not maintained its native cylindrical geometry. Therefore,

it would be helpful to use perfusion fixation of muscle tissue to maintain this geometry. One recommendation involves perfusion fixation of tissue *in vivo* before the muscle is excised. Insufficient fixation can be due to a low perfusion pressure [13], so this parameter must be carefully monitored during perfusion fixation. The addition of a 4% paraformaldehyde perfusion fixation technique could allow for more measurements to be taken. This would allow for a closer observation of impaired vasodilation and its effects on collateral enlargement. If future experimentation that includes perfusion fixation yields muscles that do not have strong enough fluorescence, antigen retrieval may be necessary. Boiling each specimen in water should result in the presentation of antigens which will yield a stronger fluorescence for functional microscopy and morphometry measurements.

Further studies could also include those which stimulate mechanoadaptation independent of arterial occlusion, so that the causal role of this process in impaired vasodilation could be evaluated. Results from this study could shed light on the exact mechanism which impairs vasodilation in a collateral after structural remodeling has occurred.

Overall, impaired vasodilation can explain some symptoms of ischemic disease, which is the highest rated cause of mortality within the United States population. The ability of the profunda femoris artery to vasodilate after collateral outward remodeling in murine hindlimbs can shed light on the mechanism behind this impairment. Further studies should include a perfusion fixation technique that maintains a vessel's native geometry paired with close observation during mechanoadaptation stimulation and inhibition. This will effectively increase the statistical power of the experiment.

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[http://www.pscho.uni-duesseldorf.de/abteilungen/aap/gpower3/download-and-register/index\\_html](http://www.pscho.uni-duesseldorf.de/abteilungen/aap/gpower3/download-and-register/index_html)

## Appendix A

**Images of each profunda at 40x**  
(Figures 6-15)

LEFT DAY 7

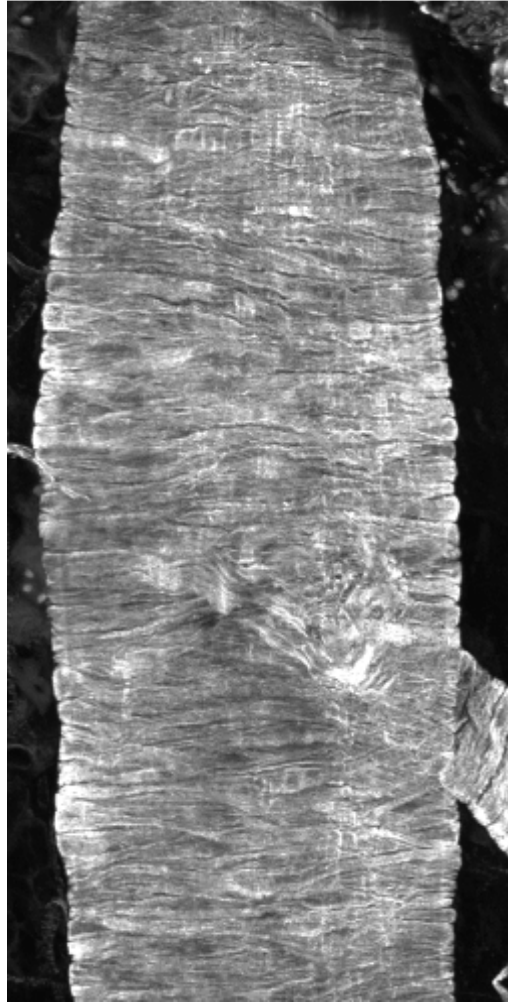
RIGHT DAY 7



LEFT DAY 7



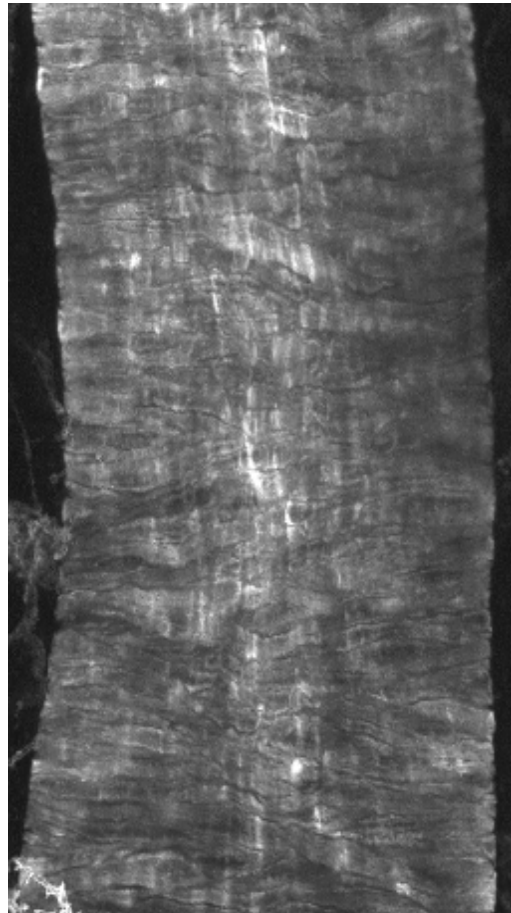
RIGHT DAY 7



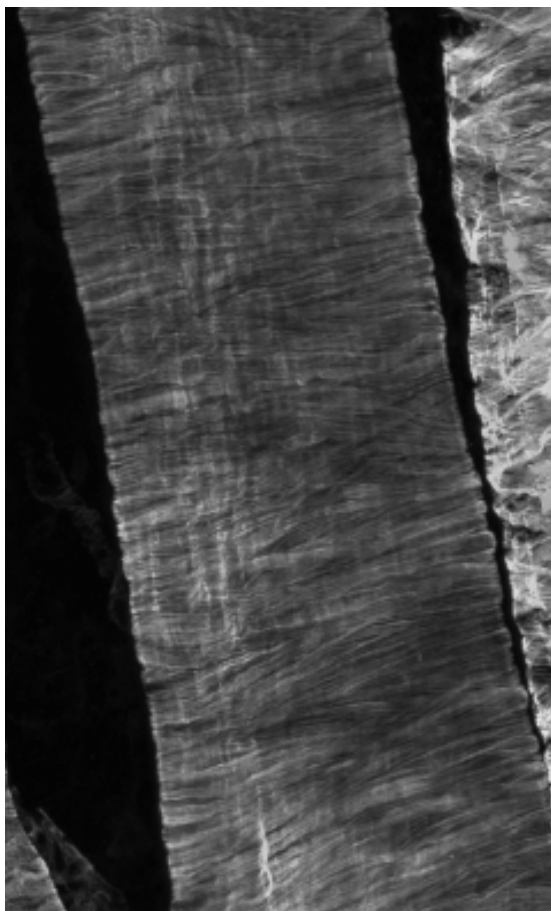
LEFT DAY 7



RIGHT DAY 7



LEFT DAY 28



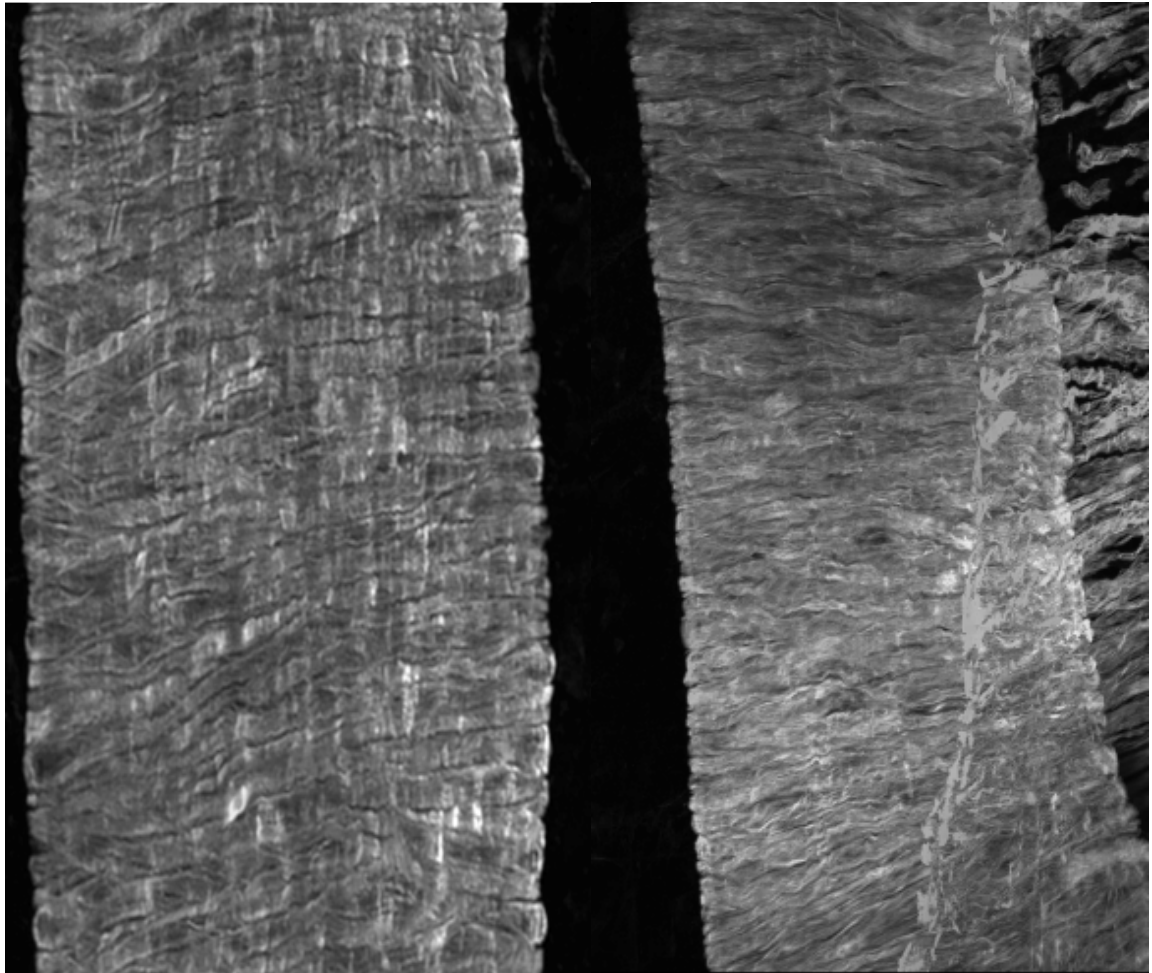
RIGHT DAY 28





LEFT DAY 28

RIGHT DAY 28



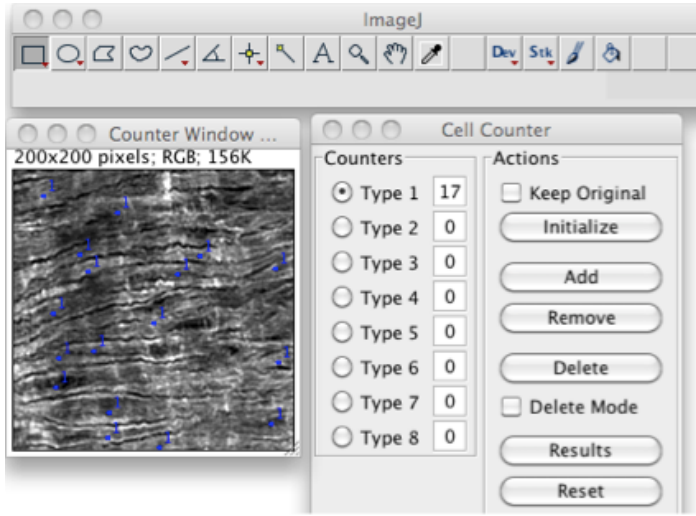
## Appendix B

### Image Analysis and Geometric Characterization

Analysis of all images was performed using ImageJ software. Each z-stack was composed of 30, 30 $\mu$ m slices. After opening the .tiff file in ImageJ, the segmented line tool was used to obtain an accurate measurement of overlap length. To measure cell length, the equation below was used.

$$\text{Circumference} = 2 * \pi * \text{Radius}$$

$$\text{Cell Length} = \text{Circumference} - \text{overlap length} \quad \text{Eq.1}$$

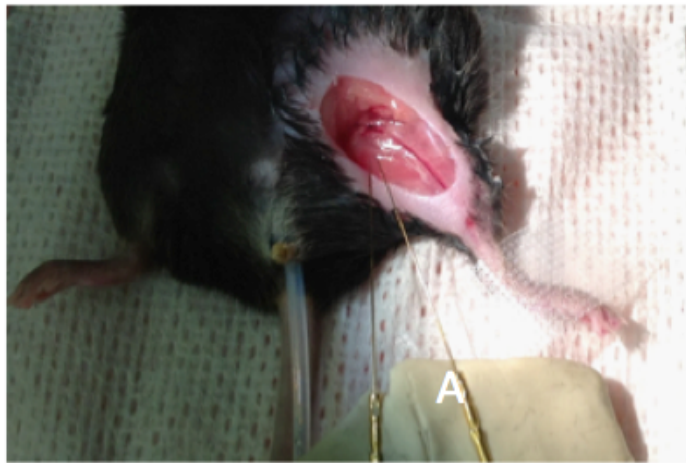


**Figure 16: ImageJ cell counter.** Each cell was assigned a number which allowed for randomization when cells were chosen to measure.

## Appendix C

### Methods

In order to model functional vasodilation, stimulation of the gracilis muscle was done in mice, activating endogenous vasodilation. After administration of anesthesia (5% isoflurane), the surgical procedure began with the application of depilatory cream to remove hair around the incision site. PBS was applied to the incision site to prevent tissue desiccation. Blunt dissection of connective tissue was done to expose the gracilis muscle. Two electrodes were placed on the muscle, which was stimulated for 90 seconds with 1mA at a frequency of 8Hz [10].



**Figure 17: Functional Vasodilation.** Two electrodes were placed on the gracilis muscle to stimulate vasodilation [10].

## **Appendix D**

### **Perfusion Fixation Background**

The current protocol for imaging vessels does not allow the muscle to maintain its cylindrical geometry which makes it difficult to image the vessel and see all of the cells. The process of fixation stops biochemical reactions from progressing and strengthens the physical stability of the tissue. Different types of fixation include heat, perfusion, and immersion. Perfusion, relevant to this study, is fixation via the circulatory system. This is done by injecting the fixation solution into the ventricles or aorta [15].

A perfusion fixation protocol in which 4% paraformaldehyde (PFA) is injected via the abdominal aorta allows for the cessation of biochemical reactions and leaves all circulatory vessels fixed in their native geometry (Appendix D).

Fixation is an immunohistochemistry process that involves the digestion of enzymes. If this digestion process is inefficient, antigen retrieval is necessary. Epitope retrieval, more specifically, is achieved by heating the specimen in question. This process is called heat induced epitope retrieval (HIER) [14]. The most common and effective method of HIER is boiling the specimen in a buffer solution.

### **Perfusion Fixation Materials and Protocol (ABCAM)**

Anaesthetic

Scissors, forceps, and clamps for surgical procedures

Small forceps with fine claws

Scalpel

Vials (5-10 ml) with lids for specimens

0.9% saline

500 ml beakers

4% paraformaldehyde, fixation solution

Gloves, eye goggles

Perfusion pump (or flask with fixative placed upside down about 150 cm above the operating table)

Short syringe needle for heart perfusion of aorta, length about 50 mm, outer diameter 1.3

- 1.5 mm

Perfusion set with drip chamber as used for intravenous blood infusions

### **Protocol (ABCAM)**

#### **Perfusion fixation through the abdominal aorta**

1. Prepare materials and animal as stated above (steps 1-3).
2. Open the abdominal cavity by a long midline incision with lateral extension, and move the intestines gently to the left side of the animal.
3. Carefully expose the aorta below the origin of the renal arteries and very gently free the aorta from overlaying adipose and connective tissues.
4. Hold the wall of the aorta firmly with fine forceps with claws about 0.5 - 1.0 cm from its distal bifurcation. Insert a bent needle close to the forceps towards the heart into the lumen of the aorta.
5. In very rapid succession:
  - a) Cut a hole in the inferior caval vein with fine scissors,
  - b) Start the perfusion and
  - c) Clamp the aorta below the diaphragm, but above the origin of the renal arteries.

When performing these manipulations accuracy and speed are essential and the fixation procedure is preferably carried out by two persons. It is particularly important to clamp the aorta rapidly after the perfusion has been started. This is most easily done by compressing the aorta toward the posterior wall of the peritoneal cavity with a finger (wear gloves) which is then replaced by a clamp. Finally, cut the aorta above the compression.

6. The kidney surface must blanch immediately and show a uniform, pale colour. The flow rate should be at least 60-100 ml/min for an adult rat. Perfuse for 3 min.

Stop the perfusion and excise and trim the tissues. Store the tissue in vials and immersion-fix in the same fixative (post fixation step) for 2 hr on ice or at 4°C.

For better results, immersion-fix overnight at 4°C.

7. The tissue is now ready for dehydration and embedding [16].

## **Appendix E**

### **Method for calculating sample size and power analysis**

Free software is available for G\*Power Free Download at:

<http://www.psych.uni-duesseldorf.de/abteilungen/aap/gpower3/download-and-register/index.html>

Enter in:

- Test family
- Statistical tests
- Type of power analysis
- Input parameters:
  - Tails
  - Effect size
  - Alpha error probability
  - Power (1-Beta)

Output is:

- Critical t
- Total sample size
- Actual power