INFLUENCE OF FIBROBLASTS ON FUNCTIONAL ARTERIOGENESIS IN A MURINE CHRONIC HINDLIMB ISCHEMIA MODEL

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PROJECT INFORMATION

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ABSTRACT

Influence of Fibroblasts on Functional Arteriogenesis in a Murine Chronic Hindlimb Ischemia Model

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Peripheral arterial occlusive disease (PAOD) occurs when there is a narrowing or blockage – usually a buildup of plaque - within the arteries that reduces blood flow to tissues which can chronic ischemia. As with most diseases, early detection and proactive treatment are important to maximize prognosis. Exercise effectively treats PAOD, but due to ischemic pain in the limbs, or intermittent claudication (IC), exercise can become painful and difficult. Due to the buildup of plaque, occlusions create an ischemic environment that changes the pressure distribution in collateral networks and increases the shear stress in transverse collaterals. Those two responses signal the beginning of arteriogenesis- the enlargement of natural bypass collaterals, which can alleviate IC and allow patients to exercise more comfortably. Arteriogenesis is controlled by macrophages and growth factors to induce proliferation in endothelium and smooth muscles cells (SMCs); enlarging arteries allow more blood to flow and oxygen to be transported. In this study, fibroblast-containing collagen constructs were transplanted under the gracilis muscle of mice with a ligated femoral artery to confirm their beneficial contribution to arteriogenesis. Collagen constructs were also found to improve functionality of muscle force production 7-days post femoral artery ligation. Though force-intensity found no statistical significant between collagen constructs and fibroblast-containing collagen constructs, they were both found to be an improvement from the baseline disease state. Force-frequency data found fibroblast-containing collagen constructs to be an improvement from the baseline disease state while being similar to the control, non-disease state. The PowerLab data acquisition unit (DAQ) and force transducer set-up is a novel method to quantify the efficacy of myogenic cells to functionally treat ischemic diseases and optimistic results of this study along with research based data acquisition improvements suggest more robust and meaningful results in a multi-operator class setting.

Key Words: PAOD, Arteriogenesis, Fibroblast, Force-Production, DAQ, Fatigue

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"It is the little details that are vital. Little things make big things happen."

- John Wooden

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Chapter 1: INTRODUCTION

Clinical Relevance

Cardiovascular (CV) disease in the leading cause of death globally and accounts for 17.3 million deaths per year, and is expected to grow to 23 million by 2030 [24]. According to the American Heart Association, CV diseases "kill more people than all forms of cancer combined" with costs totaling to be more than $320.1 billion [24]. Coronary Heart Disease (CHD) is the most common type of CV disease that kills 370,000 people and affects 735,000 in America alone [4]. Another CV disease, peripheral arterial occlusive disease (PAOD), affects 8-12 million Americans each year and evenly affects men and women [12]. The sheer number of people affected by ischemic disease and the fact that current treatments for ischemic diseases are inadequate and not applicable to the entire patient population warrant further research of therapies that mimic or promote the body's innate response to ischemia.

Peripheral Arterial Occlusive Disease and Atherogenesis

PAOD occurs when there is a narrowing or blockage within the arteries that carry blood from the heart to extremities and is usually due to the buildup of plaque along artery walls (**Figure 1**). Although PAOD equally affects men and women, the occurrence increases with age. Other risk factors for PAOD include smoking, high blood, pressure, diabetes, and high cholesterol [5]. The buildup of plaque is referred to as atherogenesis and can lead to chronic ischemia-insufficient blood flow to tissues that impairs microcirculation function. A common symptom of PAOD is pain in the leg while doing physical activity; however, up to 40% of patients do not have leg pain [5]. Due to this,

PAOD can go undiagnosed until more serious symptoms like muscle atrophy, hair loss, or non-healing ulcers [25].

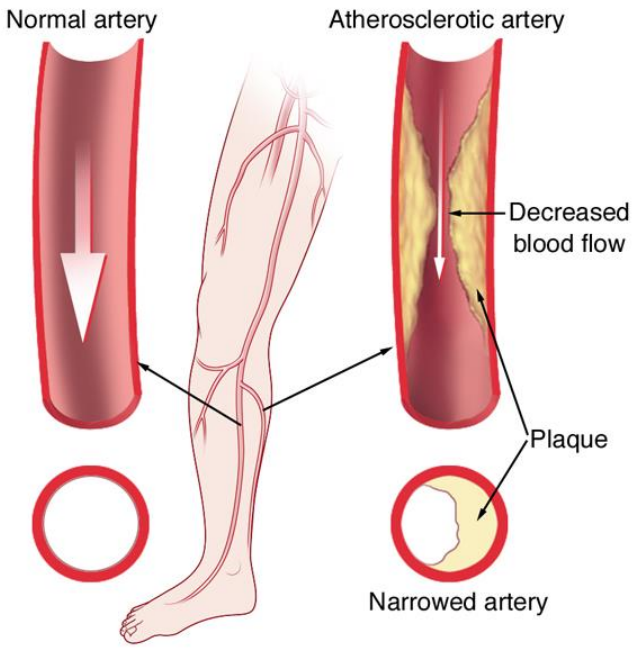

Figure 1: Peripheral Arterial Occlusive Disease
PAOD is the narrowing of peripheral arteries, more commonly legs than arms, due to atherosclerotic plaques build-up [11].

Current Treatments of PAOD

Like most diseases, early diagnosis and treatment are critical. If diagnosed early, dietary and lifestyle changes may reduce symptoms and halt further progression of the disease. Treatments also commonly include regular physical exercise, a low-fat diet, and smoking cessation. These changes can decrease cholesterol levels and lower blood pressure. Other common treatments focus on medications to manage inflammation, lipid levels, and prevent blood clots.

In more serious cases, endovascular intervention such as stenting, or bypass surgery may be required. Endovascular treatment is a minimally invasive procedure that uses catheters, wires, and even stents to treat the atherosclerotic arteries (**Figure 2**). With

advances in imaging, equipment, and endovascular expertise, endovascular treatments have become more popular due to its outpatient nature and low cost [1].

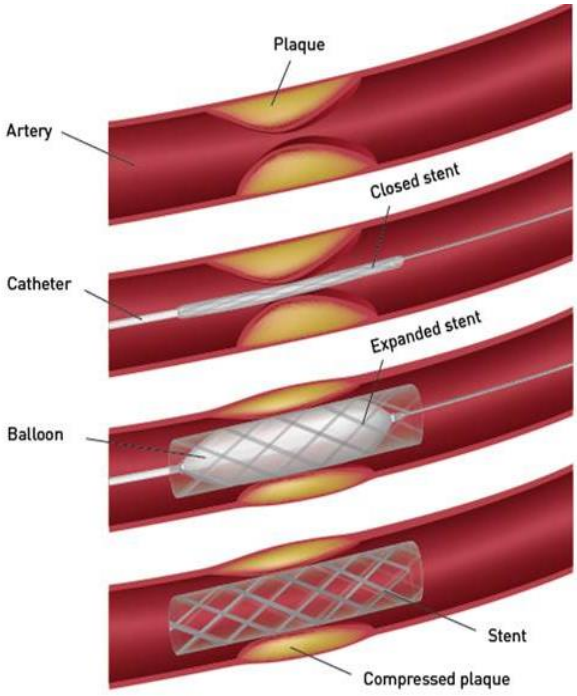

Figure 2: Stenting as an Endovascular Treatment
A stent is guided to the atherosclerotic part of the artery using catheter. Once the closed stent is in the correct area the stent is expanded using a balloon. Once fully expanded, the stent is left in place to allow better blood flow past the compressed plaque. [20].

Arteriogenesis and Vascular Remodeling

During arteriogenesis – a body's innate response to ischemia - endothelial and smooth muscle cells of existing micro vessels are stimulated to proliferate and outward remodeling occurs (**Figure 3**). This remodeling thereby enlarges natural bypasses to supply blood and oxygen to tissues downstream of the occluded artery. The presence of enlarged collaterals is a favorable prognostic factor and can increases a patient's chances of recovery [3228]. Through understanding the physiology of ischemic disease, fibroblasts have been identified as an important therapeutic target.

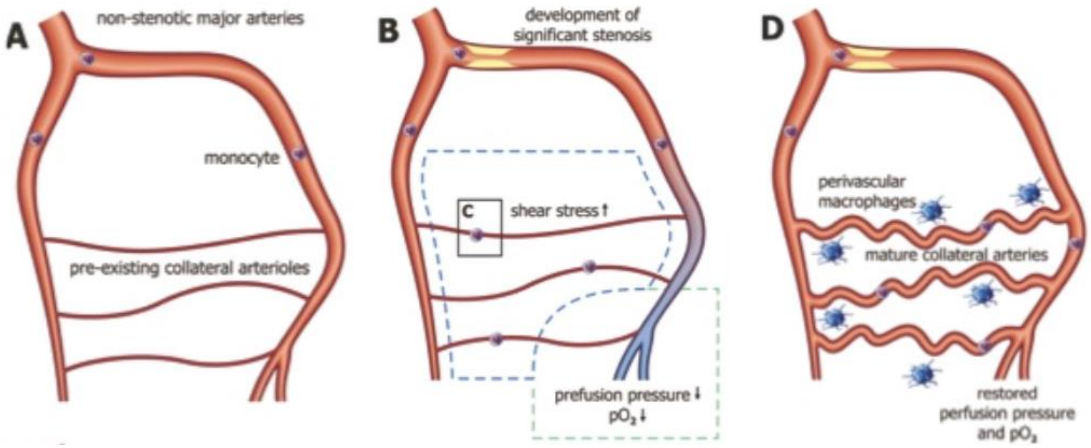

Figure 3: Overview of Arteriogenesis

Under ischemic conditions the body can naturally respond with arteriogenesis. **A** –Under normal conditions, existing collaterals are smaller in diameter. **B** – When plaque begins to build up, the pressure within the system changes causing an increase of stress in the collaterals. **D** – The changes in environment activate a series of events that increase the diameter of collaterals to restore the difference in pressure. [10].

Ischemic disease can cause insufficient blood flow to the brain, heart, or legs which can lead to stroke, myocardial infarction, or amputation due to infection, respectively; it is also accountable for millions of deaths worldwide. Ischemic diseases result from insufficient blood flow to tissues that results in necrosis, which weakens the heart, kills brain tissue, or peripheral tissue.

Ischemia is not easily treated by just one gene, protein, or cell type but scientists have found that there are synergistic benefits to therapies that combine multiple genes, growth factors, or cells. After all pharmaceutical options are exhausted, stimulation of vessel growth is considered. There are two forms of vessel growth: angiogenesis and arteriogenesis. While angiogenesis refers to the formation of new capillary networks, arteriogenesis refers to the growth of preexisting collaterals.

Arteriogenesis occurs when an arterial occlusion decreases downstream pressure and increases the shear stress in transverse collaterals. The increased shear stress activates endothelial cells causing an upregulation of cell adhesion molecules for

monocytes. The monocytes then differentiate into macrophages that secrete growth factors that stimulate the proliferations of endothelial and smooth muscle cells to increase the diameter of existing collaterals (**Figure 4**).

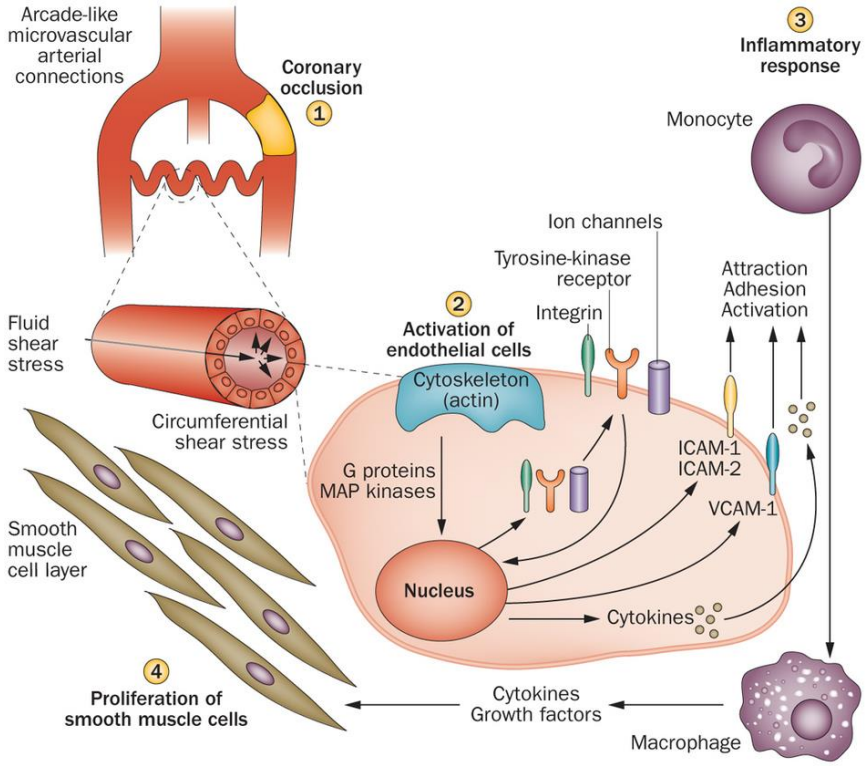

Figure 4: Molecular Mechanism of Arteriogenesis
Within the first 24 hours of an arterial occlusion, 1) changes in pressure increase shear stress within collateral arteries therefore activating endothelial cells that 2) activates signaling pathways to express multiple genes for monocyte attraction, activation and adhesion. The monocytes 3) are then transformed to macrophages that secrete growth factors to 4) stimulate smooth muscles cell proliferation [31]

Previous Work Regarding Arteriogenesis

As with many process within the body, there are a multitude of growth factors involved with the proliferation of smooth muscle cells (SMCs) in arteriogenesis [29]. Growth factors like fibroblast growth factor (FGF), transforming growth factor beta (TGF-B), platelet-derived growth factors (PDGF), insulin-like growth factor (IGF-I/II),

and monocyte chemoattractant protein-1 (MCP-1) regulate the proliferation of smooth muscle cells [16].

Although the administration of isolated growth factors stimulates arteriogenesis in animal models, they failed in clinical trials [3]. To maximize the therapeutic benefits of arteriogenesis more studies are needed to determine the most effective growth factor(s), optimal amount of the growth factor(s), when during remodeling process to administer the factors, etc [13]. Since direct inject was ineffective in answering those logistical questions, the next wave of research began transplanting cells that could naturally secret the growth factors needed for arteriogenesis [28,13].

Fibroblasts are a therapeutic target because they synthesize and maintain the matrix for vascular remodeling by controlling FGF expression [18]. Transplanted fibroblast-cell sheets effectively repair or promote vascular regeneration in damaged hearts of rats [17]. Fibroblasts and myoblasts – immature skeletal muscle cells – constructs promote arteriogenesis in murine hindlimb chronic ischemia models [6,9].

Contractile and Kinetic Properties of Muscles

Every muscle type has different distributions of slow- and fast-twitch muscle fibers; each of which produce different contractile properties and fatigue profiles. These different fibers contribute to differences in power – or force production – and fatigue properties [13]. Slow-twitch muscles are predominantly aerobic and used for endurance activities; fast-twitch muscles are predominantly anaerobic and used for sprinting or power exercise.

In general, force varies depending on the length of that muscle. Much like the elastic properties in a rubber band, increased length of muscles increases active force production and passive baseline tension (**Figure 5**).

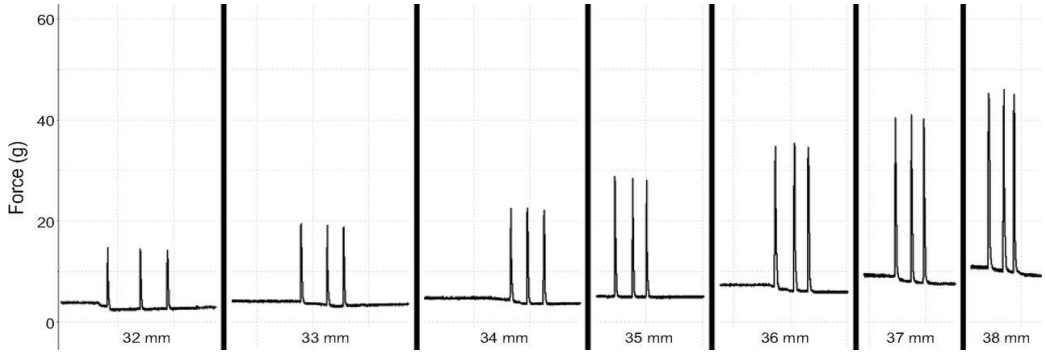

Figure 5: Length-Tension Relationship
As a muscle is stretched, elastic properties increase active force production and passive baseline tension [13].

Muscles are categorized as fast- or slow- twitch because of differences in kinetic properties. Differences in fiber composition allows fast twitch muscles to reach max force and relax quicker than slow twitch muscles (**Figure 6**).

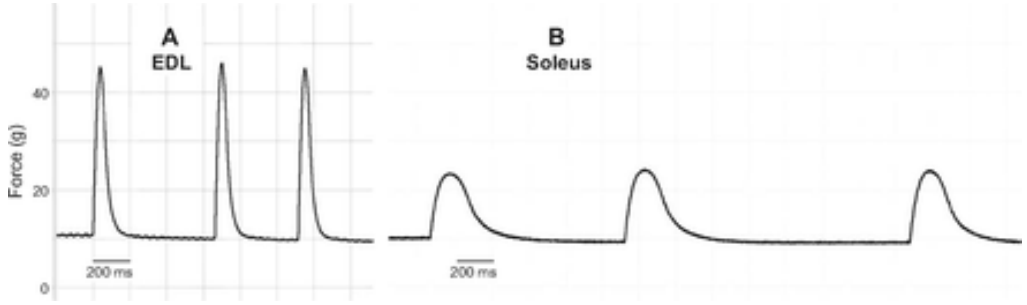

Figure 6: Twitch Kinetics
Fiber compositions allow fast twitch muscles to reach max force and relax quicker than slow twitch muscles [13].

A single stimulus produces a singular force output. Multiple stimuli – or tetanus – produce different force-frequency relationships in the different muscle types (**Figure 7**).

The tetanus response curve can be manipulated even further by decreasing or increasing the number of stimuli within a given period of time – or frequency (**Figure 8**).

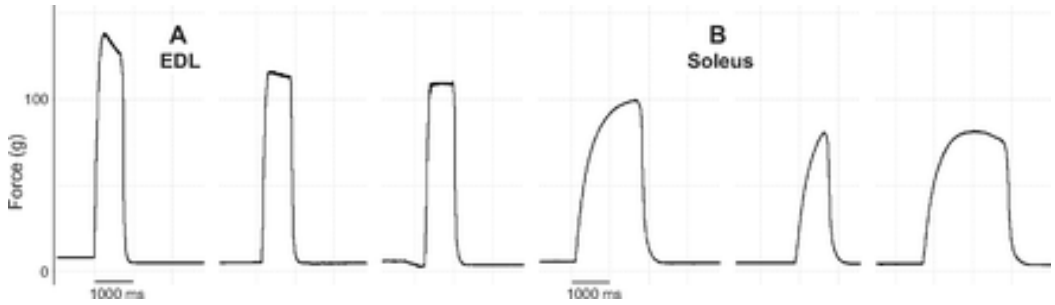

Figure 7: Tetanus Kinetics
Multiple stimuli create force-frequency curves that differ significantly in shape between fast- and slow-twitch muscles [13].

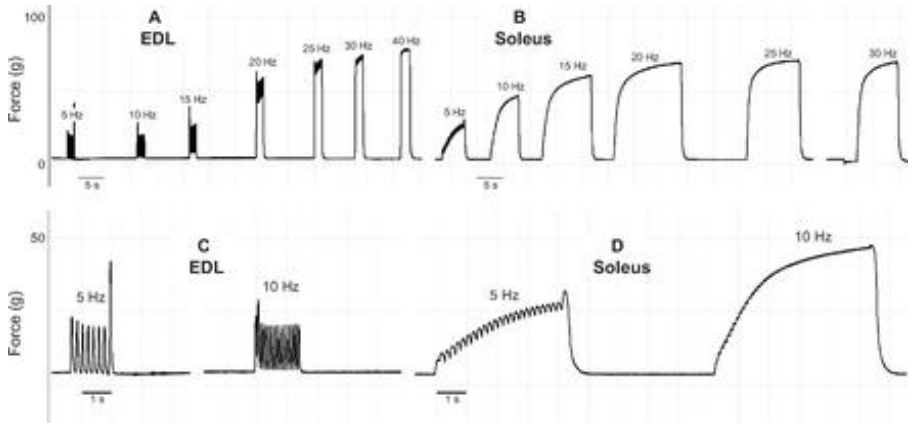

Figure 8: Force-Frequency Relationship
Due to mechanical summation, an increase in frequency generally increases the amount of force production [13].

In addition to force production measurements, another metric important to assessing muscular functionality is muscle fatigue. Fatigue occurs when there is repetitive stimulation over a longer period of time. Even though fast- and slow- twitch muscles differ on many levels, the fatigue curves produce look very similar (**Figure 9**). The quality that differentiates the two muscles types in the rate at which the force production decreases. More energy is needed and therefore is used quicker in fast twitch muscles

resulting in a faster rate of fatigue in comparison to the slower fatigue rate of slow twitch muscles.

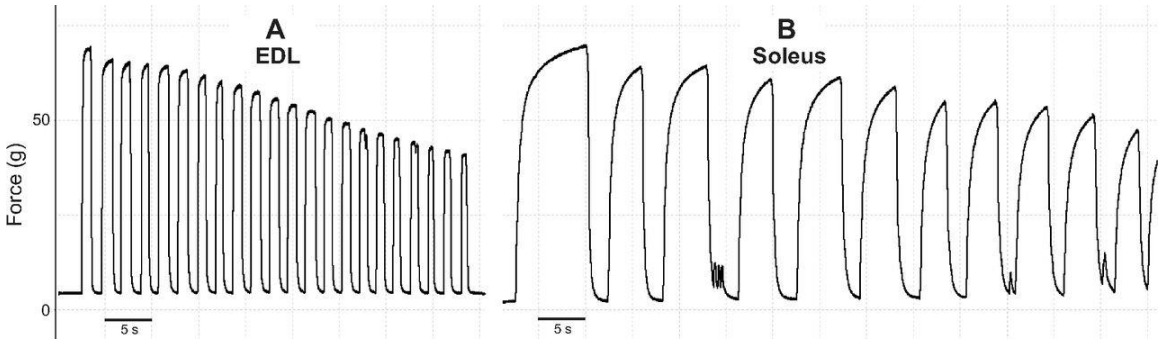

Figure 9: Fatigue Curve
In general, force production decrease with time but the rate at which it decreases differs due to energy pathways and needs of the different muscle types [13].

Specific Aims

Animals, especially rodents, are commonly used to assess numerous diseases in their natural progression along with the deviated progression due to novel therapeutic interventions. Mice are commonly used because of their convenience and inexpensiveness; they are small in size, can easily be housed and maintained, adapt well to new environments, and reproduce quickly [22]. Once a treatment is effective in-vitro at the cellular level, it must then be further tested to determine if its effectiveness in an organism.

Considering muscle function heavily depends on the oxygen and nutrients transported in blood, efficient and effective vascularization is important. Muscle function can then be assessed using force production. Excised skeletal muscle was successfully tested for isometric and eccentric force duration in muscular dystrophy murine models using a data acquisition unit (DAQ) [23]. Muscular exhaustion – or fatigue – was tested

in live mice using a forced treadmill protocol that measured total distance and speed at the point of total exhaustion occurred [19].

Since myoblast have already been found to promote arteriogenesis, a class of 15 students tried to assess the functional efficacy of fibroblast-collagen constructs using force production measurements. The fibroblast-collagen construct was statistically different from all groups except for the collagen-only construct. Therefore, the specific aim of this project is to:

1. Repeat class experiment but improve confidence in results by decreasing statistical variation due to multiple operators and operating in a more controlled environment.
2. Refine protocol to efficiently produce significant results given multiple operators and strict time restraints.

Chapter 2: MATERIALS AND METHODS

Animal Housing and Care

Male and female ICR mice along with female Swiss Webster mice were used in the procedures in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of California Polytechnic State University, San Luis Obispo. Mice were housed in the University Vivarium and were monitored daily for food, water and additional enrichment factors (cage bedding, mouse house, and tunnel tube).

Mouse Preparation

Mice were anesthetized in an induction chamber using 5% isoflurane in oxygen flowing at 3-5 $l \cdot min^{-1}$. They were then subcutaneously administered buprenorphine analgesic (0.075 $mg \cdot kg^{-1}$). After the initial anesthetization, mice were transferred to a heating pad on a surgical slate and administered 1-3% isoflurane flowing at 0.8-1.0 $l \cdot min^{-1}$ through a nose cone. The heating pad was set to maintain a core body temperature of 35ºC for the duration of the procedure using a rectal temperature probe. The hair on the lower, ventral region of the mouse was removed prior to surgery with trimming clippers and depilatory cream. Veterinary ointment was then applied to the corneas to prevent desiccation. The entire mouse preparation protocol can be seen in **Appendix A**.

Femoral Artery Ligation

To model peripheral arterial occlusion (PAO), a femoral artery ligation was performed in the hindlimb of a murine animal model. The entire procedure was

performed under aseptic conditions to reduce contamination and infections. The initial incision was made on the medial potion of the thigh and stopped at the abdominal wall (**Figure 10A**). Using blunt dissection, surrounding connective tissue was removed and the epigastric fat pad was retracted and reveal the neurovascular bundle. Connective tissue above the bundle was gently removed to isolate the femoral artery; once it was isolated 6-0 silk suture was used to ligate the femoral artery between the epigastric and popliteal branches **(Figure 10B**). The entire femoral artery ligation protocol can be seen in **Appendix A**.

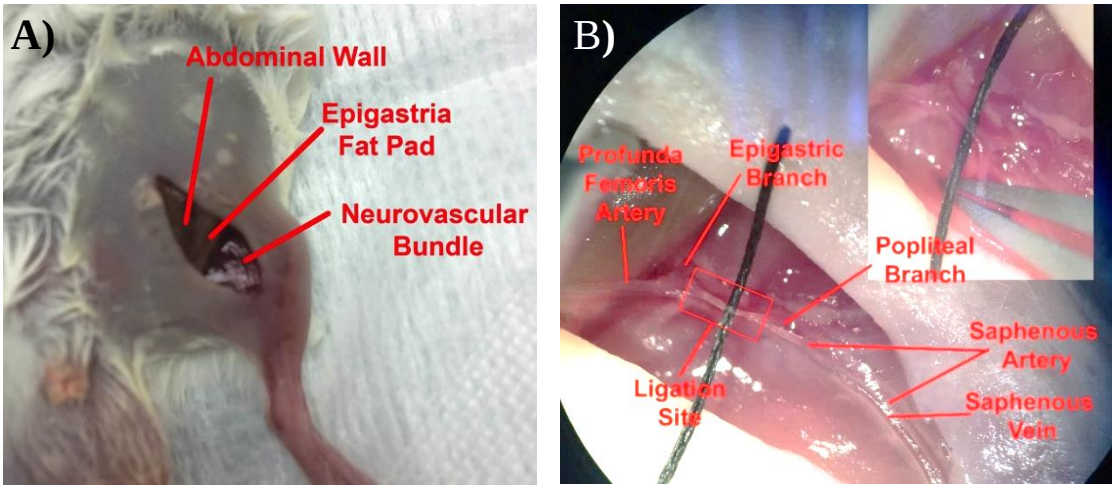

Figure 10: Femoral Artery Ligation.
A – The initial incision was made on the medial aspect of the femur to the abdominal wall to expose the epigastric fat pad and neurovascular bundle. **B** – The femoral artery was ligated between the epigastric and popliteal branches.

Fibroblast Construct Transplantation and Suture

A premade pocket under the gracilis anterior muscle was created using blunt dissection to house the collagen construct. The entire construct was then gently placed beneath the muscle (**Figure 11A**). To prevent desiccation the entire surgical site was irrigated with sterile phosphate buffered saline (PBS). The incision was then closed using 7-0 polypropylene monofilament suture (**Figure 11B**). The mouse was then

subcutaneously administered buprenorphine analgesic (0.075 $mg \cdot kg^{-1}$) again. Mice were then returned to the University Vivarium to be monitored daily using ambulation scoring until walking normally. The entire femoral artery ligation protocol can be seen in **Appendix A**.

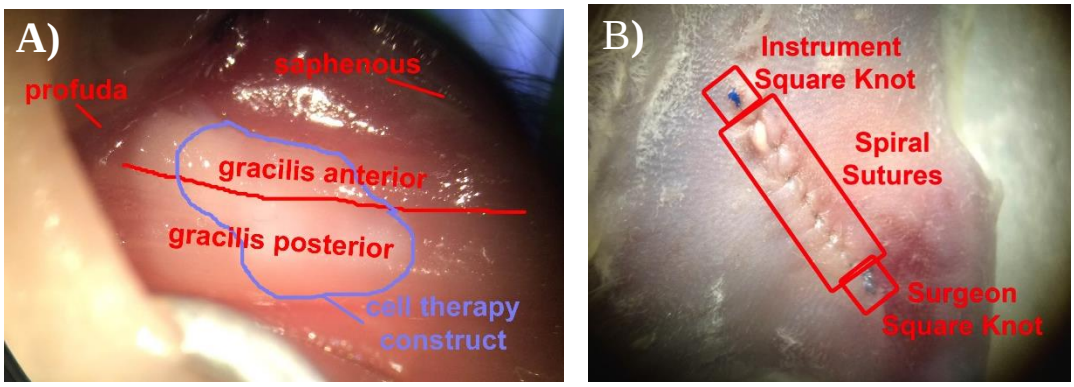

Figure 11: Fibroblast Construct Transplantation.
A – The entire fibroblast construct was gently tucked under the gracilis muscle. **B –** The surgical incision is then sutured closed.

The surgical groups used for this study can be found in **Table 1** below. Surgical group 1 consisted of mice that were ligated and blunt dissected under the gracilis muscle but did not receive a construct. Surgical group 2 consisted of mice that were ligated and blunt dissected to receive a construct with no fibroblasts. Surgical group 3 consisted of mice that were ligated and blunt dissected to receive a construct with fibroblasts.

Table 1: Experimental Design and Surgical Groups

Surgical Groups	Number of Mice	Description
1	4	Ligated, Dissected, no construct
2	5	Ligated, Dissected, construct with no cells
3	5	Ligated, Dissected, construct with fibroblasts

Force Production and Gracilis Muscle Harvest

Seven days post-surgery, arteriogenesis was functionally tested using force production measurements as seen in **Appendix B**. This was achieved using blunt dissection to dorsally reflect the sciatic nerve, which was stimulated to contract the gracilis muscle (**Figure 12A**). Once the nerve was reflected using parafilm, a curved current electrode was attached. Then the Achilles tendon is exposed and silk suture is tied to the end of the tendon (**Figure 12B** and **Figure 12C**). The suture is used to connect the Achilles to a force transducer (Protocol in **Appendix C**). Measurements were taken for force-intensity, force-frequency and fatigue.

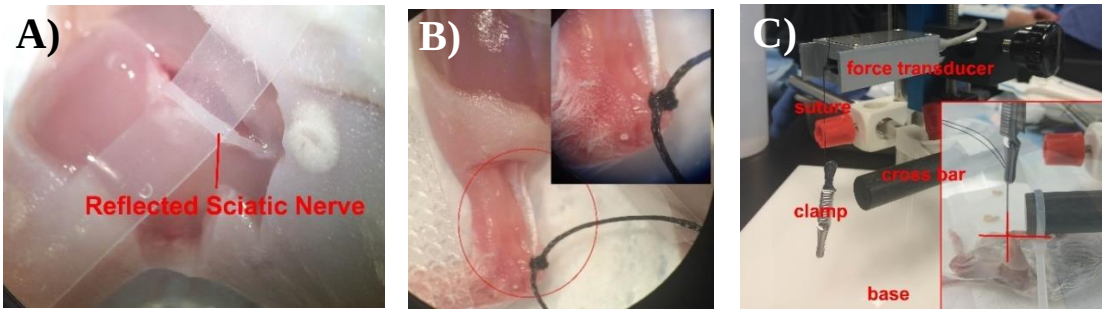

Figure 12: Force Production Set-Up.
A – The sciatic nerve is reflected using parafilm for ease of access. **B –** The Achilles tendon is then exposed and silk suture is knotted to the end so it can be connected to the force transducer. **C –** The whole force production set up is constructed to quantitatively measure an aspect of arteriogenesis.

Once measurements were taken, sodium nitroprusside (SNP) was applied to the gracilis muscle to induce maximal vasodilation. After maximal dilation, the gracilis was fixed in-situ for 15 minutes using paraformaldehyde (4% PFA) and covered with plastic wrap to prevent desiccation. After fixation, the muscle was removed from surrounding tissue and placed in more PFA overnight at 4^{o}C. Once 24 hours have passed, they were washed using and store in PBS at 4^{o}C until stained.

Staining and Imaging

Alpha smooth muscle-actin (α-SMA) was used to stain and visualize the collateral network throughout the muscle. A working solution of α-SMA (1:200) was prepared along with saponin (0.1%) and bovine serum albumin (BSA, 2%) in PBS. The muscle was incubated in the stain for 3 nights. After the staining period, they were washed in a saponin (0.1%) and PBS mixture three times and in PBS once. Finally, they were mounted on glass slide. This staining protocol is in **Appendix D**.

A widefield fluorescent microscope was sued to visualize and image the muscle using 550nm excitation and 570nm emission. Images of the stem, midzone, and re-entry were captured at 4x or 10x magnification. Gracilis imaging protocol is in **Appendix E**.

Data and Statistical Analysis

The Data Pad function in Lab Chart Reader was used to obtain force production and integral measurements and then exported to Microsoft Excel according to the protocol in **Appendix F**. Microsoft Excel was then used to graphically display data while ImagesJ was used to measure vessel diameters. Differences in force production, force intensity, and fatigue along with percent change in diameter were determined by ANOVA tests, a $p<0.05$ was used to indicate significance.

Chapter 3: RESULTS

The potential functional contribution of fibroblasts was tested by transplanting fibroblast-containing collagen constructs into a mouse model with a ligated femoral artery. The three groups tested were: ligated with no construct in the pocket, ligated with collagen construct in the pocked, and ligated with a fibroblast-containing collagen construct in the pocked. Force production measurements were taken regarding stimulation intensity, stimulation frequency, and fatigue, and results are presented below. The control group consists of the non-ligated leg of each mouse.

Force-Intensity

In general, across all treatment groups, force production increased with increased stimulation intensity (**Table 2**). Overall, the pocket group was lower than the collagen and fibroblast groups (**Figure 13**). With regards to various stimulation intensities, the control group was greater than the pocket group between 0.75 and 4.5 mV.

Table 2: Muscle Force Production with Increasing Intensity

Intensity (mV)	Force Production (mN/mg)			
	Pocket	Collagen	Fibroblast	Control
0.25	0.04 ± 0.04	0.06 ± 0.04	0.00 ± 0.00	0.18 ± 0.10
0.50	0.06 ± 0.06	0.24 ± 0.14	0.29 ± 0.27	0.54 ± 0.14
0.75	0.16 ± 0.11	0.49 ± 0.24	0.54 ± 0.39	0.94 ± 0.11
1.00	0.28 ± 0.15	0.67 ± 0.33	0.59 ± 0.38	1.06 ± 0.10
1.25	0.36 ± 0.16	0.75 ± 0.37	0.69 ± 0.36	1.14 ± 0.10
1.50	0.43 ± 0.16	0.77 ± 0.37	0.79 ± 0.36	1.17 ± 0.11
1.75	0.46 ± 0.17	0.77 ± 0.35	0.83 ± 0.36	1.20 ± 0.13
2.00	0.48 ± 0.17	0.78 ± 0.34	0.86 ± 0.35	1.17 ± 0.15
2.25	0.50 ± 0.18	0.83 ± 0.32	0.93 ± 0.34	1.21 ± 0.12
2.50	0.52 ± 0.18	0.89 ± 0.30	0.98 ± 0.33	1.28 ± 0.13
2.75	0.54 ± 0.18	0.93 ± 0.29	1.00 ± 0.33	1.30 ± 0.13
3.00	0.56 ± 0.19	0.97 ± 0.29	1.03 ± 0.32	1.34 ± 0.12
3.25	0.59 ± 0.19	1.01 ± 0.28	1.10 ± 0.35	1.31 ± 0.10
3.50	0.59 ± 0.19	1.05 ± 0.28	1.08 ± 0.33	1.38 ± 0.12
3.75	0.62 ± 0.19	1.08 ± 0.30	1.07 ± 0.34	1.43 ± 0.12
4.00	0.64 ± 0.19	1.04 ± 0.28	1.06 ± 0.34	1.45 ± 0.12
4.25	0.59 ± 0.19	1.07 ± 0.27	1.05 ± 0.35	1.51 ± 0.13
4.50	0.63 ± 0.19	1.18 ± 0.34	1.04 ± 0.34	1.47 ± 0.12

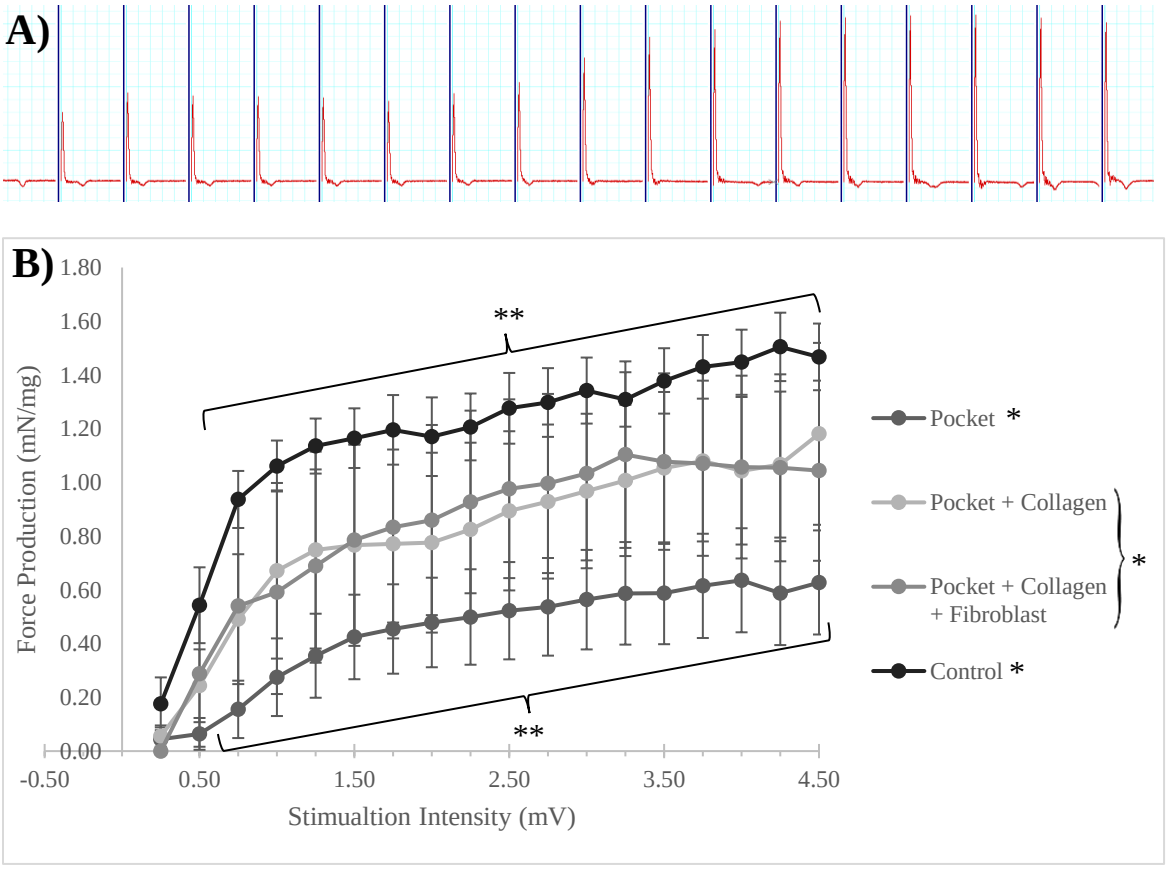

Figure 13: Muscle Force Production with Increasing Intensity

Force produced in measured for twitch recruitment of four sub-groups at increasing stimulation intensities: .25, .50, .75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.25, 3.50, 3.75, 4.00, 4.25, and 4.50 mV. A – Raw data trace of force production using a data acquisition unit that stimulated the sciatic nerve and measured force using a force transducer attached to the Achilles tendon of a mouse. B – Averaged normalized muscle force production, using individual gastrocnemius weights, for each treatment group at increasing stimulation intensities (n=14 control, n=4 pocket, n=5 collagen, and n=5 fibroblasts). Data are presented as mean ± SE. * denotes $p < 0.05$ for pocket vs collage/fibroblast vs control and ** denotes $p < 0.05$ for pocket vs control at 0.75mV to 4.5 mV.

Force-Frequency

In general, the control group and the fibroblast group were greater than pocket group (**Table 3**). In most cases the stimulation frequency of 50 Hz produced the greatest force production; excluding the collagen group which had higher force production in the 5Hz and 10 Hz frequency groups (**Figure 14**). At 50 Hz stimulation frequency only the fibroblast group was significantly greater than the pocket group. At 2.5 Hz and 5 Hz stimulation frequency, only the control group was significantly greater than the pocket

group. In all the other stimulation frequency categories, no significant difference was found among the groups.

Table 3: Muscle Force Production with Increasing Frequency

Freq. (Hz)	Force Production (mN/mg)			
	Pocket	Collagen	Fibroblast	Control
2.5	0.64 ± 0.20	1.34 ± 0.49	1.25 ± 0.39	1.80 ± 0.15
5.0	0.68 ± 0.21	1.33 ± 0.48	1.26 ± 0.40	1.80 ± 0.16
10.0	0.67 ± 0.21	1.03 ± 0.34	1.34 ± 0.33	1.25 ± 0.22
20.0	0.69 ± 0.21	1.07 ± 0.42	1.45 ± 0.33	1.31 ± 0.24
50.0	1.02 ± 0.47	1.29 ± 0.52	2.34 ± 0.63	1.88 ± 0.40

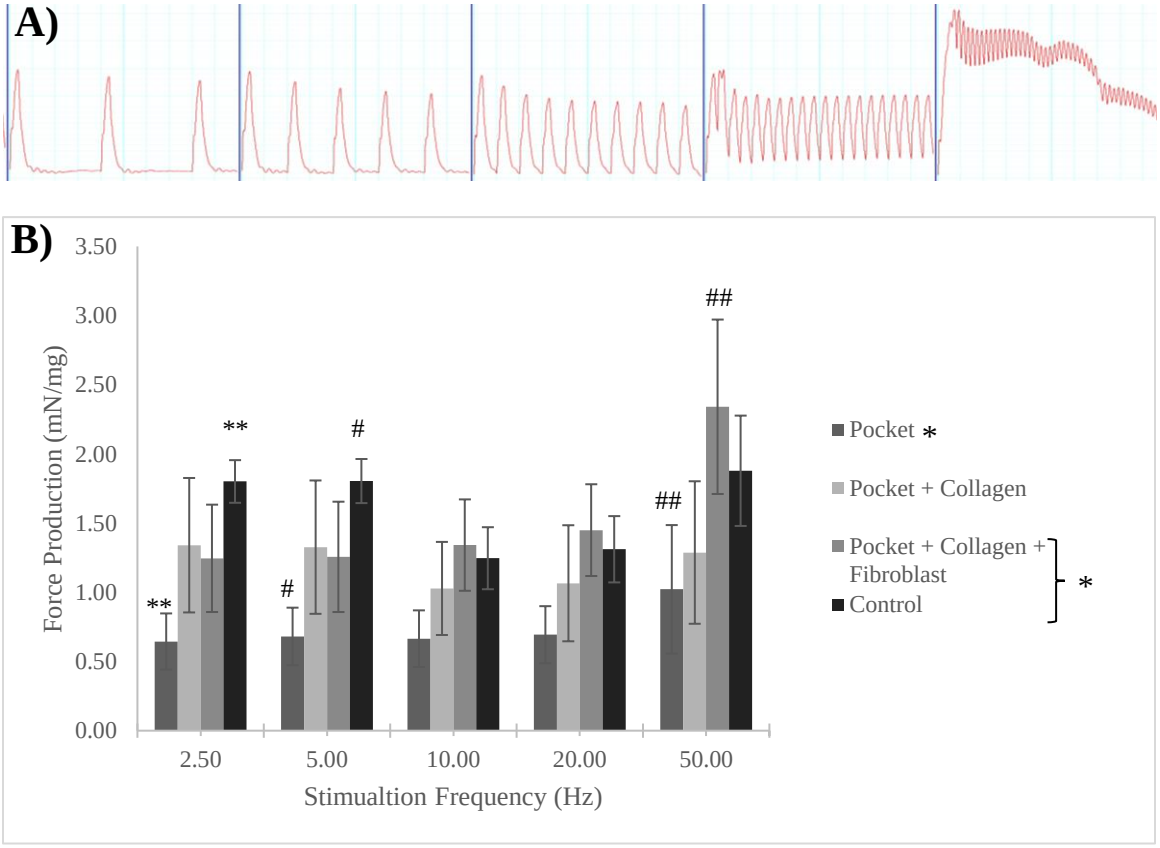

Figure 14: Muscle Force Production with Increasing Frequency
Force produced in measured for twitch recruitment of four sub-groups at increasing stimulation frequencies: 2.50, 5.00, 10.00, 20.00, and 50.00 Hz. A – Raw data trace of force production using a data acquisition unit that stimulated the sciatic nerve and measured force using a force transducer attached to the Achilles tendon of a mouse. B – Averaged normalized muscle force production, using individual gastrocnemius weights, for each treatment group at increasing stimulation frequencies (n=14 control, n=4 pocket, n=4 collagen, and n=4 cells). Data are presented as mean ± SE. * denotes $p < 0.05$ for pocket vs fibroblast/control, ** denotes $p < 0.05$ for pocket vs control at 2.5 Hz, # denotes $p < 0.05$ for pocket vs control at 5.0 Hz, and ## denotes $p < 0.05$ for pocket vs fibroblast at 50 Hz.

Fatigue

Overall, pocket was found to have the smallest integral and shortest half-time (**Table 4**: Muscle). With regards to integrals, the collagen group was found to be significantly greater than the pocket group. No significant difference was found regarding time to half maximum force production (**Figure 15**).

Table 4: Muscle Fatigue - Time to Half and Integral of Maximum Force Production

Surgical Group	Pocket	Collagen	Fibroblast	Control
Abs. Integral (mV/s)	17.48 ± 10.78	60.90 ± 7.64	45.94 ± 10.72	44.17 ± 8.14
Time to Half (s)	6.31 ± 3.29	11.81 ± 3.27	13.45 ± 5.60	11.47 ± 3.65

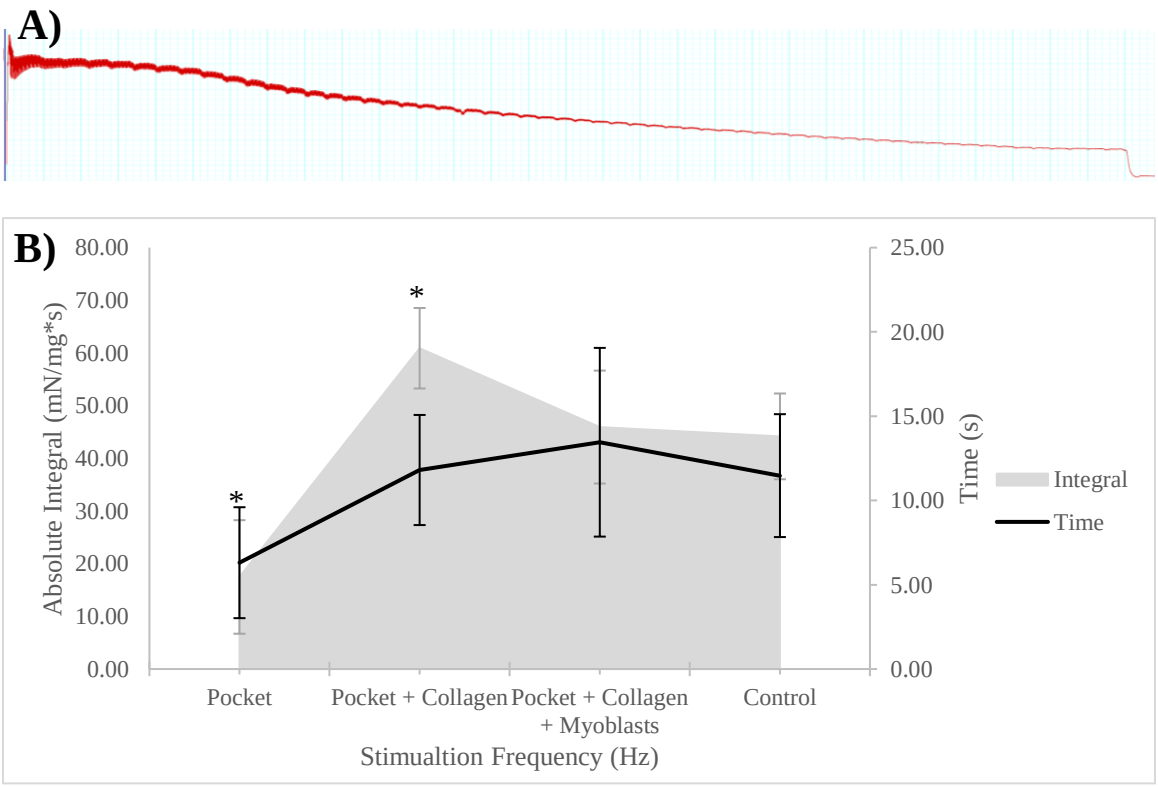

Figure 15: Time to Half and Integral of Maximum Force Production
Force produced in measured using maximum stimulation intensity and frequency for 30 seconds. The time it takes to go from maximum force production to half of that is also show. A – Raw data trace of force production using a data acquisition unit that stimulated the sciatic nerve and measured force using a force transducer attached to the Achilles tendon of a mouse. B – Averaged normalized muscle force production, using individual gastrocnemius weights, for each treatment group at (n=9 control, n=2 pocket, n=3 collagen, and n=4 cells). Data are presented as mean ± SE. * denotes $p < 0.05$ for pocket vs collagen with regard to absolute integral.

Overall, the collagen and control groups have a smaller percent change than the fibroblast group which was smaller than the pocket group (**Table 5**). With regard to the control, fibroblast, and collagen groups, the rate of percent change was similar before 10 second and then leveled off at differing rates (**Figure 16**).

Table 5: Percent Change in Fatigue Force Production

Time (s)	Force Production (%)			
	Pocket	Collagen	Fibroblast	Control
0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
1	79.2 ± 15.7	89.6 ± 3.9	83.8 ± 2.2	73.2 ± 5.8
2	69.5 ± 26.2	84.0 ± 4.4	80.2 ± 5.0	70.2 ± 5.8
3	60.7 ± 34.0	77.8 ± 5.4	75.5 ± 8.0	66.5 ± 5.3
4	56.4 ± 33.8	71.2 ± 6.9	70.6 ± 10.5	62.6 ± 4.7
5	51.8 ± 33.2	64.4 ± 8.1	65.7 ± 11.3	57.9 ± 4.4
6	48.2 ± 30.3	58.8 ± 9.6	60.9 ± 11.5	54.0 ± 4.4
7	42.0 ± 27.5	54.3 ± 10.5	56.0 ± 11.1	51.1 ± 4.6
8	36.0 ± 23.4	50.7 ± 11.1	51.2 ± 10.9	49.3 ± 4.7
9	31.3 ± 20.5	48.2 ± 11.6	47.7 ± 11.1	48.0 ± 4.9
10	28.3 ± 18.3	46.1 ± 11.5	45.0 ± 11.1	46.8 ± 5.1
11	25.2 ± 16.6	45.1 ± 11.0	36.9 ± 7.1	45.7 ± 5.3
12	21.9 ± 14.8	44.1 ± 10.5	33.9 ± 6.7	45.0 ± 5.5
13	19.7 ± 13.2	43.1 ± 9.9	34.3 ± 8.6	44.4 ± 5.6
14	17.5 ± 11.8	42.2 ± 9.3	33.5 ± 10.0	44.1 ± 5.6
15	16.5 ± 10.1	41.2 ± 8.7	31.4 ± 10.3	43.6 ± 5.6
16	14.5 ± 9.8	39.9 ± 8.4	30.0 ± 10.7	43.3 ± 5.5
17	12.9 ± 8.8	38.9 ± 7.9	28.6 ± 11.2	42.7 ± 5.6
18	11.8 ± 7.9	40.7 ± 4.5	26.7 ± 11.2	41.8 ± 5.5
19	10.6 ± 7.4	40.1 ± 3.5	24.4 ± 11.1	41.1 ± 5.6
20	9.7 ± 6.7	38.4 ± 3.2	23.8 ± 11.1	40.4 ± 5.7
21	8.5 ± 6.4	37.3 ± 2.8	23.1 ± 11.6	39.4 ± 5.7
22	7.7 ± 5.9	35.8 ± 2.7	22.3 ± 12.4	38.7 ± 5.8
23	6.6 ± 5.8	34.5 ± 2.5	21.6 ± 12.5	37.8 ± 5.8
24	6.1 ± 4.8	32.8 ± 2.9	20.5 ± 12.9	36.9 ± 5.7
25	5.2 ± 4.1	31.7 ± 2.9	20.0 ± 13.2	36.1 ± 5.7
26	4.4 ± 3.8	30.2 ± 3.1	24.5 ± 17.4	35.4 ± 5.8
27	3.7 ± 3.3	29.2 ± 3.1	23.8 ± 16.9	34.7 ± 5.7
28	3.1 ± 2.9	28.1 ± 2.9	23.4 ± 17.3	34.1 ± 5.8
29	2.6 ± 2.6	26.9 ± 3.2	23.1 ± 17.5	33.4 ± 5.8

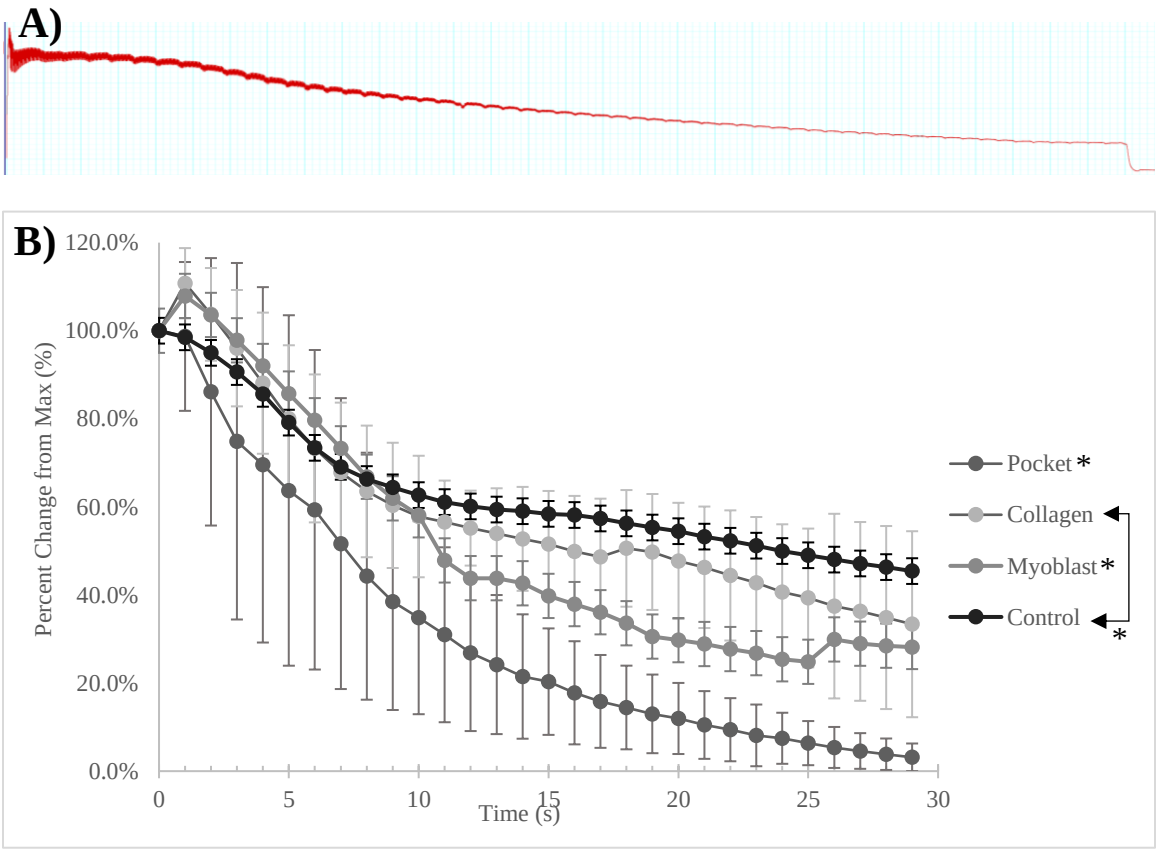

Figure 16: Percent Change in Fatigue Force Production

Force produced in measured using maximum stimulation intensity and frequency for 30 seconds. The percentage is calculated using the average force production in the given time window divided by the max force production in the 0 to 1sec time interval. A – Raw data trace of force production using a data acquisition unit that stimulated the sciatic nerve and measured force using a force transducer attached to the Achilles tendon of a mouse. B – Percent change in force production over a 30sec time interval (n=9 control, n=2 pocket, n=3 collagen, and n=4 cells). Data are presented as mean ± SE. * denotes $p < 0.05$ for pocket vs myoblast vs collagen/control.

Profunda Femoris Diameter

Overall, the collagen and fibroblast groups had the largest percent change in diameter in the midzone (**Table 6**). Although the fibroblast group saw largest change in diameter in each zone it was not statistically significant from the other surgical groups (**Figure 17**). In the midzone, the fibroblast group was greater than the pocket group.

Table 6: Percent Change in Diameter of Collateral Networks

Surgical Group	Reentry	Midzone	Stem
Pocket	19.4 ± 5.6%	14.3 ± 7.2%	31.6 ± 8.8%
Collagen	22.6 ± 9.0%	38.1 ± 10.1%	35.3 ± 6.8%
Fibroblast	35.2 ± 6.4%	46.6 ± 14.1%	38.4 ± 8.0%

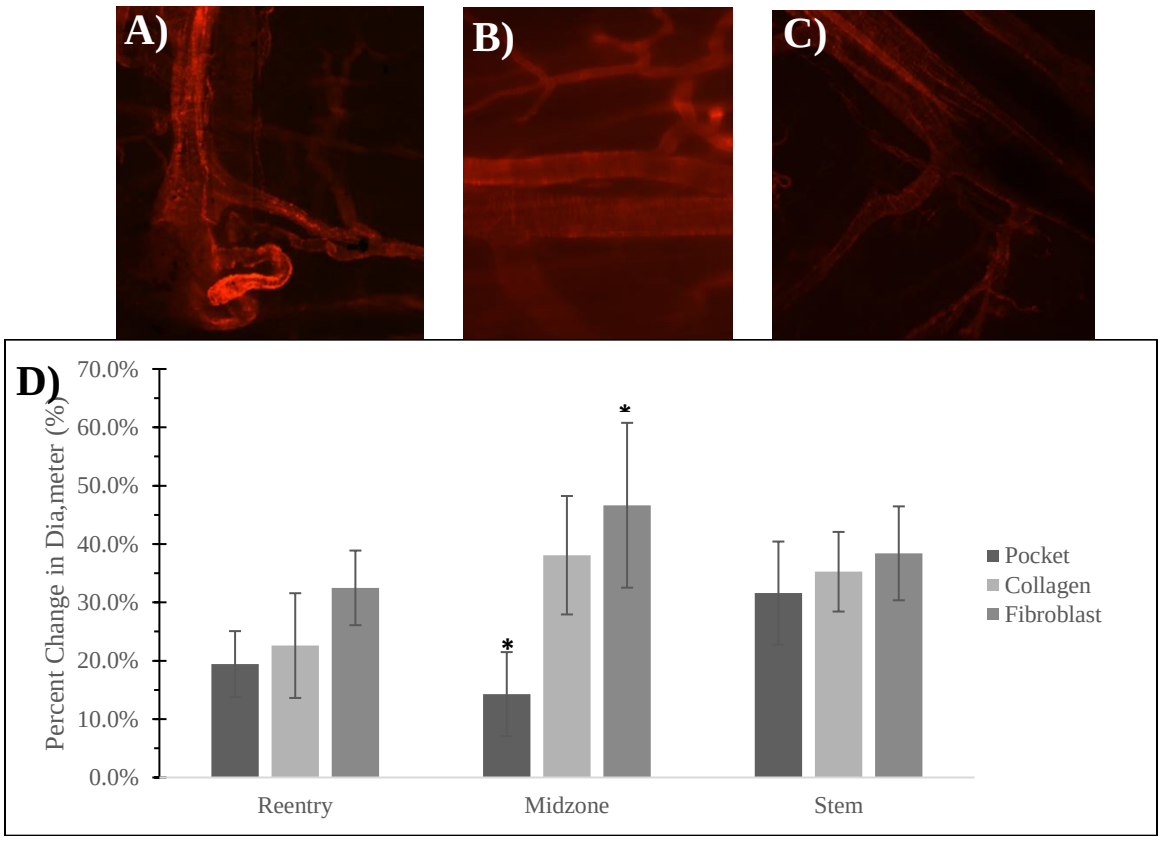

Figure 17: Percent Change in Diameter comparing Surgical Groups

Diameters for each zone in the collateral network was compared within each surgical group with respect to their control. A – Raw photo of the Profunda or stem zone at 4x magnification. B – Raw photo of the midzone at 10x magnification. C – Raw photo of the Saphenous or reentry zone a 4x magnification. D – Percent change in diameter for each surgical group with respect to their control (n=15 control, n=4 pocket, n=5 collagen, and n=6 cells). Data are presented as mean ± SE. * denotes $p < 0.05$ for pocket vs fibroblast in the midzone.

Chapter 4: DISCUSSION

Clinical Significance

Since PAOD is a chronic disease with general symptoms that disproportionately affect an already vulnerable group – the elderly – it is a challenging disease to address. Even though exercise is effective in prolonging the disease's onset, this disease can also make it painful to exercise. Other options include oral vasoactive compounds and surgical stenting [26]. Oral vasoactive compounds are cheap but can have secondary side effects that require close monitoring. Stenting is a more invasive treatment and carries the dangers of inserting synthetic, foreign materials in to the body.

These results from this study proposes another treatment by localizing arterial remodeling and using materials made by the body. The transplantation of fibroblast-containing collagen constructs under collateral networks of ligated mice was found to promote collateral enlargement in the midzone. Though this study found that collagen constructs functionally improves muscle force production with regard to intensity and frequency, it was not able to differentiate between collagen-only constructs and fibroblast-containing collagen constructs.

Future Work

Since the 1980s, previous research focused on characterizing the factors contributing to arteriogenesis [2]. The research in the past couple decades have tested those factors as single growth factors and have transplanted cells that are known to secrete those factors, to determine which ones are more effective or how much is needed to be effective [27]. The next wave a research is to assess the functionality of all these treatments. One of the first functionality assessments tested muscular exhausting or fatigue using a treadmill.

More recent studies use DAQs to obtain muscle force production measurements. Starting in the early 2010s, muscles were excised, stored in specialized solutions, and connected to two force transducers to measure force production [23,30]. It was only in the past few years that studies have directly attached the mouse to DAQs and electrically stimulated them to obtain force production measurements to assess cardiovascular disease [7].

DAQ set-ups inherently allow more consistency in data collection than observational studies and therefore it can decrease some variability. This study in particular, has demonstrated the utility in assessing the functional viability for therapeutic treatments. It not only compares the proposed treatment to the disease state without treatment but it can also produce control or non-disease state results. Furthermore, multiple metrics for muscle functionality –force production, force intensity and fatigue – can comfortably and accurately be tested within one session, unlike some previous methods. Since excising a muscle can cause damage and affect data, it is safer to excise it after collecting force production measurements.

Limitations

Considering the complexity of this study there were many logistical limitations that resulted in variations in many external factors.

First, to keep this project manageable, constructs were made by students whose focus were to create the collagen constructs. Due to this, surgeries were scheduled around when constructs could be made; this resulted in non-randomized data collection. Also, due to previous commitments of other students, the constructs had to made by multiple people.

Additionally, considering how many projects were going on at the time, the mouse selection was first-come, first-serve. This resulted in two mice types being used: Swiss Webster

and colony mice. Inherently, the colony mice have more variation and contained both male and female mice while the Swiss Websters' were only female. Sex influences the vascular response due to impaired vasodilation [8].

Procedural Improvements

After performing the protocol numerous times, there are many tips and reminders to ensure an effective and efficient data collection session. The biggest suggestion would require operators to familiarize themselves with contractile and kinetic properties of muscle to understand why these metrics are used and recognize the results produced [13].

Throughout the study, the one error that created the longest delay is the slippage of the suture connecting the muscle to the force transducer. In order to prevent the suture from slipping off the Achilles tendon, cut the tip of the calcaneus to hold the suture knot in place. To prevent the suture from slipping out of the force transducer, use a hook- rigging mechanism instead of the clamp mechanism used now. This could be achieved by knotting the open end of the suture to create a loop that can be secured to a hook rather than pinched between clamp.

After analyzing force-intensity production data many intensity intervals were found to be redundant (see **Appendix G**). In order to save time collecting and analyzing the data, the 18 intervals at 0.25mV increments can instead be cut in half by stimulating every 0.5 mV from 0.5mV to 4.5mV.

Previous research indicates that the gastrocnemius muscle consists mostly of fast-twitch muscle fibers [13]. This suggest data obtain would more closely resemble that of an EDL muscle. Previous research has found that EDL-like muscles have a very unique force-frequency

curve (**Figure 18**). Considering this, less points before 10 Hz and more points between 15Hz and 30 Hz can better predict the force-frequency relationship in the gastrocnemius muscle.

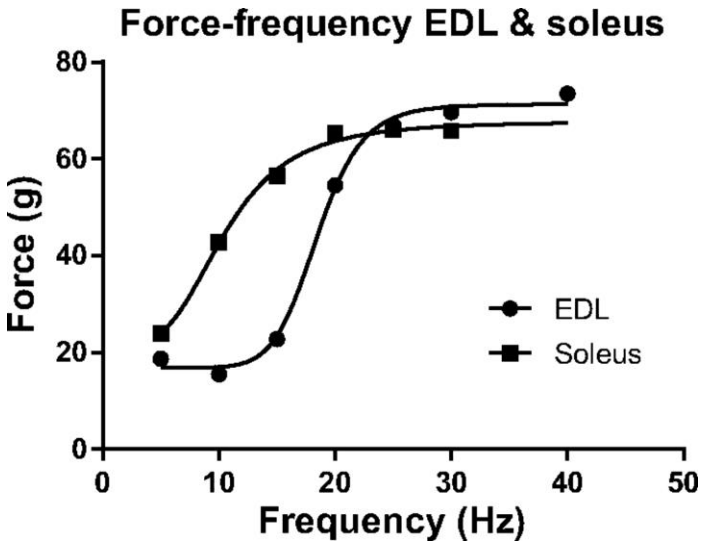

Figure 18: Force Frequency Curve of EDL Muscle
Considering the gastrocnemius muscle is mostly composed of fast twitch muscles it most likely that its force-frequency curve will resemble that of the EDL [13].

With regard to fatigue, the most difficult task was identifying a way to intuitively present and accurately analyze the data. No statistically significant differences or trends were found in comparing area under the curve nor time to half of the max force. An educational active learning protocol effectively depicted fatigue as a percentage of decline force over a period of time (**Figure 19**). By taking an average of force production, in bin sizes of a second, from 0s to 30s of the fatigue data collect a more intuitive graph can be depicted and more accurate statistics can be taken.

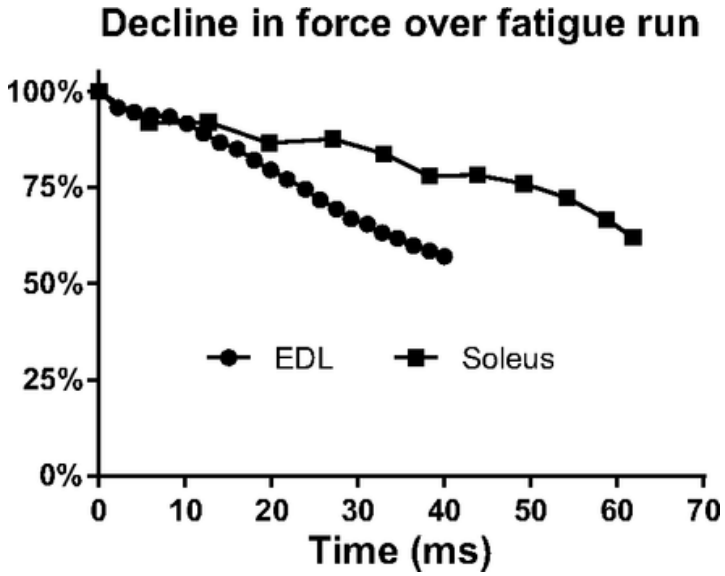

Figure 19: Rate of Force Production During Fatigue

Maximum force production declines with time. Slow twitch muscles decline at a slower rate than fast twitch muscles [13].

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APPENDIX

Appendix A: Restraint, Ligation, and Suturing Protocol for Arteriogenesis in a Mouse Model

CAL POLY SAN LUIS OBISPO	BMED 545: Cell Transplantation and Therapeutics	DATE 02/11/15	PAGE 1	OF 2
		WRITER AAS	NO.	REV. 6
TITLE **Restraint, Ligation, and Suturing Protocol for Arteriogenesis in a Mouse Model**				

1.0 PURPOSE

This protocol serves as a reference checklist to perform a surgical modification to a mouse model of peripheral arterial occlusion (PAO) and deliver a cell therapy to promote arteriogenesis by 1) properly restraining and intraperitoneally injecting anesthetic to minimize trauma and pain, 2) skillfully performing a skin incision under a stereomicroscope to minimize amount of stitches needed, 3) ligating the femoral artery to model PAOD, 4) inserting a cell therapy construct to promote arteriogenesis and 5) accurately suturing the incision to reduce infection.

2.0 Equipment

- Stereomicroscope
- Gloves (2 sets)
- Hot pack
- Bench covers
- Spray bottle of 70% isopropyl alcohol (IPA)
- Spray bottle of Nolvasan
- Syringe (1ml/10ml)
- Surgical tape
- Gauze Sponges (4x4 & 2x2)
- Surgical drapes (20x20 & 16x20)
- Depilation cream (Veet)
- Ophthalmic ointment
- Cotton Swabs
- Standard pattern forceps
- Curved iris scissors
- Dumont #7 forceps
- Needle holders
- Prolene 7-0 Blue monofilament suture
- C-1 13 mm 3/8 taper suture needle
- Silk suture (1-2 inches)
- Cell therapy construct
- Retractor
- Autoclave tape

3.0 Methods

3.1 Basic Rules

a. *Always set down forceps and scissors with tips up.*
b. *Hold instruments in any way to perform movement without contorting body and compromising stability.*
c. *Maximize exposure and minimize trauma.*
d. *Keep back straight/good posture to get through long surgeries.*
e. *Saline. Saline. Saline. When you pull head out of scope, add saline.*
f. *Do not go over sterile fields.*

3.2 Sterilized Instruments

- Surgical drape (18-20inches square).
- Use autoclave tape to close tray and wrap.

3.3 Sterile pack

- Prolene suture
- Silk ligature
- 3 4x4 gauze sponge
- 4 cotton swabs
- surgical drape 16x20inches

3.4 Open and activate hot pack

- Weight mouse _____grams

3.5 Scruff Restrain and Injection

- Grab base of mouse's tail with dominant hand.
- With fingertips (thumb & index) pinch scruff with non-dominant hand.
- Invert: pinch base tail with ring finger and use middle finger to support its back.
- Angle animal/nose downward.
- Insert needle at a 30o angle (bevel side up) (**Figure 1a**).
- Pull back on syringe to confirm vacuum.
- IP inject fluid/anesthesia. SubQ inject analgesia.
- Paw pinch.

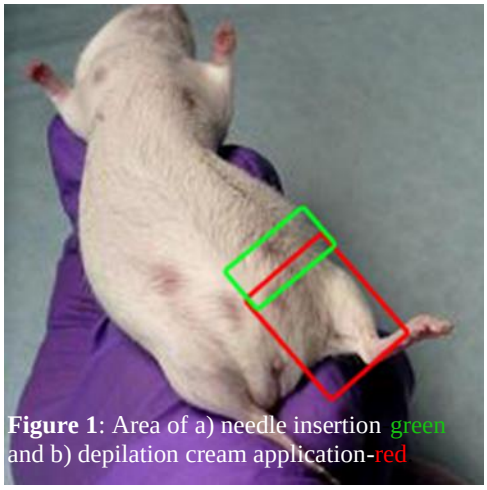


Figure 1: Area of a) needle insertion green and b) depilation cream application-red

3.6 Depilation

- Tape down mouse's feet on 4x4 sponge gauze.
- Rub cream going against the grain (**Figure 1b**).
- Let rest for a couple minutes.
- Remove with gauze sponge. Remove excess off with Nolvasan.
- Paw pinch.
- Apply eye ointment and punch ear hole.
- Move to fresh bench cover (heating pad).

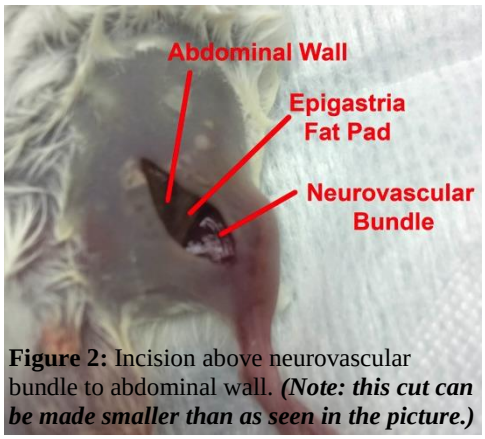


Figure 2: Incision above neurovascular bundle to abdominal wall. ***(Note: this cut can be made smaller than as seen in the picture.)***

3.7 Sterile set up

- Open sterile tray away from bench cover.
- Open and dump contents of sterile pack.
- Wash hand up to elbow with disinfectant soap
- Put on isolation mask and cap.
- Put on sterile gloves.
- Prep saline dish. (ask partner)
- Move and put sterile drape over mouse up to front paws.
- Cut small window. Feel for hind limb.

3.8 Skin Incision

- Tent skin with standard pattern forceps.
- With iris scissors, cut incisions perpendicular to tented skin and above femoral vein and artery.
- Keep cutting until past epigastria fat pad near the abdominal wall (~1 cm), see **Figure 2**.
- Blunt dissect connective tissue using Dumont #7 forceps.

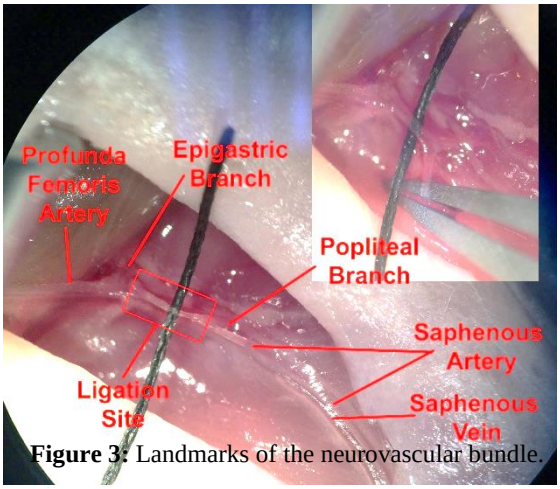


Figure 3: Landmarks of the neurovascular bundle.

3.9 Ligation

- Blunt dissect to move epigastric fat pad.
- Gently remove connective tissue over neurovascular bundle.
- Separate artery from vein.

  Note: Be aware of landmarks (**Figure 3**).
- Ligate upstream from popliteal and downstream from the epigastric.
- Isolate artery with silk suture.
- Tie off artery (2 knots).

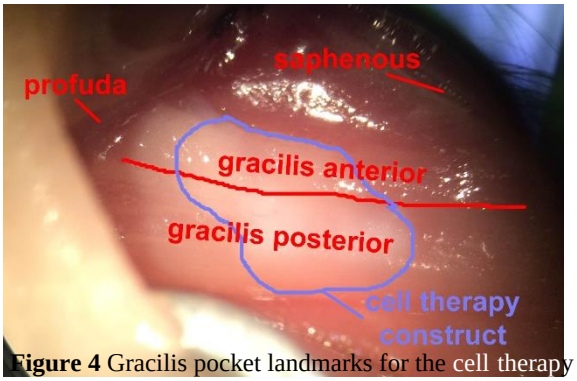


Figure 4 Gracilis pocket landmarks for the cell therapy construct.

3.10 Gracilis Pocket (Cell therapy construct)

- Lightly jiggle muscle to see gracilis separation
- Grab muscle gently at top margin.
- Blunt dissect just underneath muscle.
- Create small pocket for construct.
- Reflect back pocket and carefully stuff collagen gel underneath adductor muscle: see **Figure 4**

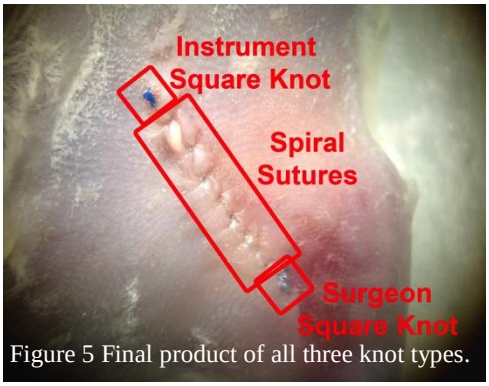


Figure 5 Final product of all three knot types.

3.11 Suturing

DO NOT OVER TIGHTEN

- Using graph forceps pull skin back push suture through skin locked needle holders.
- Surgeon square knot at bottom end of incision (6 knots).
- Spiral sutures up the length of incision.
- Instrument square knot (6 knots)
- Cut off excess suture; see **Figure 5** for final product.

Date_____ **Hindlimb Ischemia Surgery - Resection** Initials _____

Mouse Information

Mouse Information

DOB: _____

Sex: _____

Tag: _____

Genotype/strain: _____

Cage: _____

Materials

Sterilize- autoclave or flash autoclave

____1. forceps (2)
____2. fine forceps (2)
____3. ultrafine forceps (1)
____4. fine scissors (1)
____5. microscissors spring loaded (1)

Pre-sterilize in autoclave

____6. cotton gauze (2)
____7. cotton swabs (12)
____8. 6.0 silk suture (2 x 1-inch)
____9. needle holder (1)

Obtained in surgery suite

____10. sterile Petri dish w/ sterile saline
____11. sterile gloves
____12. sterile 7.0 prolene suture
____13. heat-cautery
____14. FST heat pad w/ rectal probe
____15. heat pad
____16. recovery bin & weigh boat
____17. depilatory cream
____18. non-sterile cotton swabs
____19. non-sterile cotton gauze
____20. isolation mask & cap
____21. analgesic (Buprenorphine)

Surgery preparation

____22. Spray surgery area with Nolvasan
____23. Weigh animal in weight boat
____24. Place animal in anesthesia box
____25. Open the oxygen cylinder and set anesthesia-machine flow meter to ~3 $l \cdot min^{-1}$
____26. Anesthetize animal w/ 5% isoflurane
____27. Affix non-rebreathing circuit to bench-top with tape
____28. Reduce flow rate to 0.5-1.0 $l \cdot min^{-1}$ and the isoflurane to 1-3%
____29. Apply ear tag high on left ear
____30. Lay animal supine with nose in nose-cone
____31. Shave hair on the right hindlimb & lower abdomen with clippers
____32. Remove excess hair with depilatory cream
____33. Spray right hindlimb with Nolvasan
____34. Return animal to anesthesia box
____35. Apply 4x4 gauze sponge to heat pad to protect animal from excessive heat
____36. Affix non-rebreathing circuit to surgery table w/ chemistry clamp
____37. Lay animal supine on circulating heat pad w/ nose in nose-cone
____38. Insert rectal probe and set thermo-controller to 37°C
____39. Apply veterinary ointment to eyes to avoid drying during procedure
____40. Apply veterinary ointment to anus and place rectal probe ~1cm into anus to monitor core-body temperature

Surgery

____41. Make a small incision on the middle, medial aspect of the left thigh
____42. Extend the incision up to the abdominal wall
____43. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure
____44. Use cautery to remove fat pad overlying femoral a-v pair & cauterize epigastric a-v pair
____45. Blunt dissect the femoral artery-vein pair from the nerve starting just upstream to the muscular branch & extending to half-way between the knee & ankle
____46. Tie off the saphenous a-v pair with 6.0 silk suture, halfway between the knee & ankle
____47. Tie off the femoral a-v pair with 6.0 silk suture, halfway just upstream to the muscular branch

____48. Grasping the distal ligature, use the cautery & microscissors to remove the a-v pair up to the upstream ligature
____49. Gently rest cotton swabs on hemorrhage sites
____50. Use 6.0 polypropylene suture to close the skin
____51. Make a small incision on the middle, medial aspect of the right thigh
____52. Extend the incision up to the abdominal wall
____53. Blunt dissect the subcutaneous connective tissue to maximize surgical exposure

Post-Surgical

____54. Give the animal an subcutaneous injection of buprenorphine (0.075mg/kg)
____55. Place the animal in the recovery bin, on a blue bench cover, above a heat pad and allow to recover
____56. Turn flow meter down to 0, turn off isoflurane, and close the oxygen cylinder
____57. Indicate surgery on cage card

Notes

_____

_____

_____

_____

_____

_____

_____

Appendix B: Force Production and Atrophy of the Gastrocnemius and Immunohistology of the Gracilis Muscle

CAL POLY SAN LUIS OBISPO	BMED 545: Cell Transplantation and Therapeutics	DATE 02/04/15	PAGE 1	OF 2
		WRITER AAS	NO.	REV. 3
TITLE **Force Production and Atrophy of the Gastrocnemius and Immunohistology of the Gracilis Muscles.**				

1.0 PURPOSE

This protocol serves as a reference checklist to assess the efficacy of treating a ligated artery mouse with the fibroblast cell construct by 1) dissecting and reflecting the sciatic nerve for ease of stimulation of a muscle contraction and gently dissecting the Achilles tendon to measure muscle force production of the gastrocnemius muscle, and 2) removing the gracilis to stain and assess artery growth, and 3) removing the gastrocnemius muscle to weigh and evaluate muscle atrophy.

2.0 Equipment

- Depilation cream (Veet)
- Cotton Swabs
- Saline
- Gauze Sponges (4x4 & 2x2)
- Stereomicroscope
- Standard pattern forceps
- Curved iris scissors
- Parafilm (1/8”-1/4” strip)
- Silk suture (1-2 inches)
- Force transducer set up
- Plastic cab tie
- Curved electrode (current mA)
- Surgical tape
- Power lab/Laptop
- Paraformaldehyde
- Spray bottle of Nolvasan
- Spray bottle of 70% isopropyl alcohol (IPA)
- Syringe (1ml/10ml)
- Dumont #7 forceps
- Retractor

3.0 Methods

3.1 Depilation

- Lay mouse on stomach and tape.
- Apply Veet on the left side (medial and laterally/dorsal and ventral).

3.2 Sciatic Dissection

- Lay stomach down (dorsal)
- Make incision hip and knee.
- Blunt dissection tissue using scissors.
- Cut laterally to maximize exposure and find triangle of adipose (**Figure 1**).
- Lift up adipose with #7 forceps and blunt dissect gap.

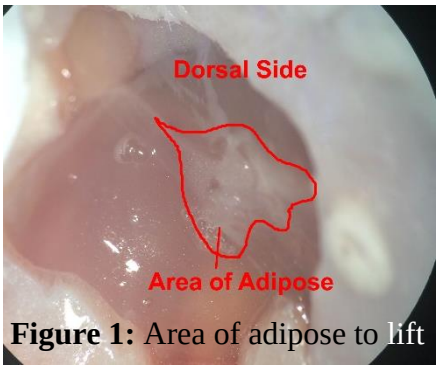


Figure 1: Area of adipose to lift

- Look for sciatic nerve (white) – clearly visible outside of scope too.

3.3 Sciatic Reflection

- Grab parafilm strip.
- Place forceps under nerve from the top and grab parafilm.
- As seen in **Figure 2**.
- Apply saline then cover with plastic wrap.

Figure 2: Reflected sciatic nerve with parafilm

3.4 Achilles Dissection

- Using graph forceps and iris scissors make skin incision right above heel.
- Gently blunt dissect and cut with iris scissors.
- Cut of skin to improve visibility. Go farther than heel.
- Blunt dissect with #7 forceps.
- Tie suture (2x) around tendon (see **Figure 3**).
- Do NOT cut until connected to force transducer.

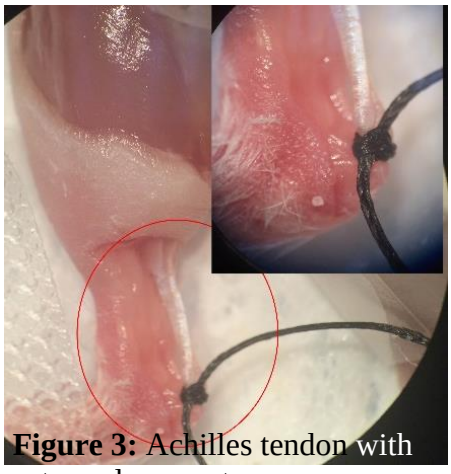


Figure 3: Achilles tendon with suture placement.

*3.5 Force Transducer Setup (see **Figure 4**)*

- Place mouse stomach up.
- Use cable to attach hind paw to cross bar.
- Grab suture with standard patterns.
- Attach suture to force transducer and cut tendon with iris scissors.
- Using #7 pull sciatic nerve to wrap hook electrode around it.
- Tape down end of electrode.
- Connect VNC cable to top output port (+).
- Hook up PowerLab (as in 460)

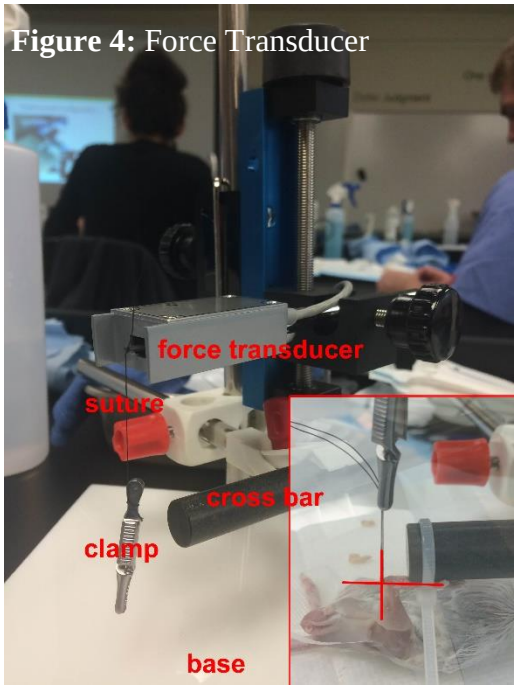
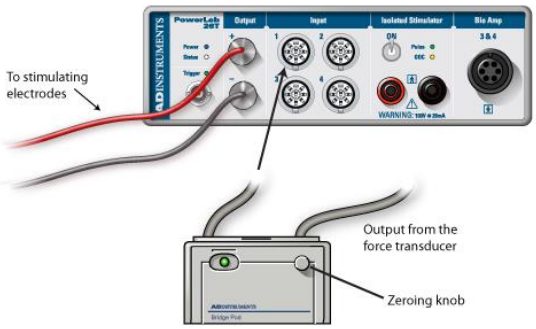


Figure 4: Force Transducer

3.6 Transducer Optimization

- Center pinhole of transducer has to be right above Achilles and gastrocnemius.
- 90o angle in all planes.
- Electrode → PowerLab → Computer → Lab Chart



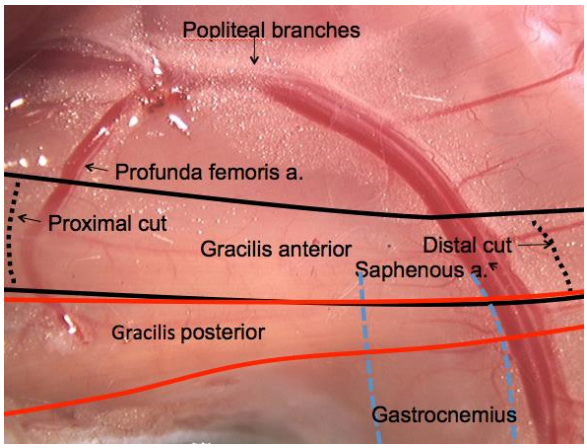


Figure 5: Gracilis dissection excision landmarks.

3.7 Gracilis Exposure

- Place stomach up on surgical stage.
- Make incision (is same place as ligation).
- Extend using iris scissor blunt dissection.
- Blunt dissect fat pad out of way.
- Apply paraformaldehyde on gracilis muscle.
- Cover with plastic crap.
- Let sit for 20 minutes.

3.8 Gracilis Dissection Excision (anterior)

- Use gauze sponge to pick up extra paraformaldehyde fixative.
- Start dissecting near gracilis pocket top.
- "Tunnel" above and below gracilis to free the whole muscle.
- Blunt dissect under the anterior gracilis.
- Blunt dissect outside saphenous and profunda.

Note: Do NOT go deep. Gracilis anterior is fairly thin.

- Cut muscle with iris scissors outside of saphenous and profunda (**Figure 5**).
- Put muscle in centrifuge tube for post fixation.

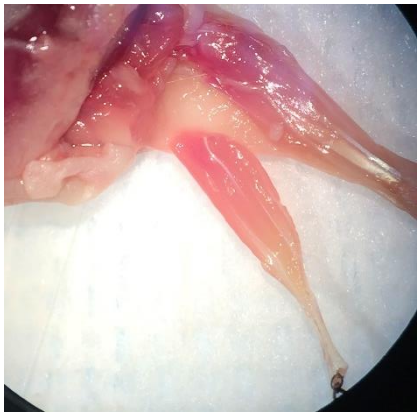


Figure 6: Separated Gastrocnemius muscle.

3.9 Gastrocnemius Dissection Excision

- Cut off skin between Achilles and gracilis to expose gastrocnemius.
- Can just peel away from hind limb using tendon.
- Cut adductor muscle on top of gastrocnemius.
- Blunt dissect and cut using iris scissors to separate muscle. (See **Figure 6**).
- Weight.

Appendix C: PowerLab Optimization Protocol

LabChart® Student Protocol

Required Equipment

- LabChart 7 software
- PowerLab Data Acquisition Unit
- Bridge Pod
- Force Transducer
- Small weight between 5–50 grams
- Manipulator/Micropositioner and clamps
- Ring stand
- Chemistry clamp
- Silk suture
- Petri dish
- One mouse
- Saline
- Small millimeter ruler
- Tape
- Muscle Stimulation Equipment
- Vascular Clamp

Procedure

Equipment Setup and Calibration

1. Make sure the PowerLab is turned off and the USB cable is connected to the computer.

2. Connect the Force Transducer cable to the back of the Bridge Pod. Connect the Bridge Pod to Input 1 on the front panel of the PowerLab (Figure 7). Connect the Stimulating Electrodes to the output on the front panel of the PowerLab. Follow the color scheme in Figure 7. *Note that newer PowerLab Systems do not rely on the Bridge Pod.*

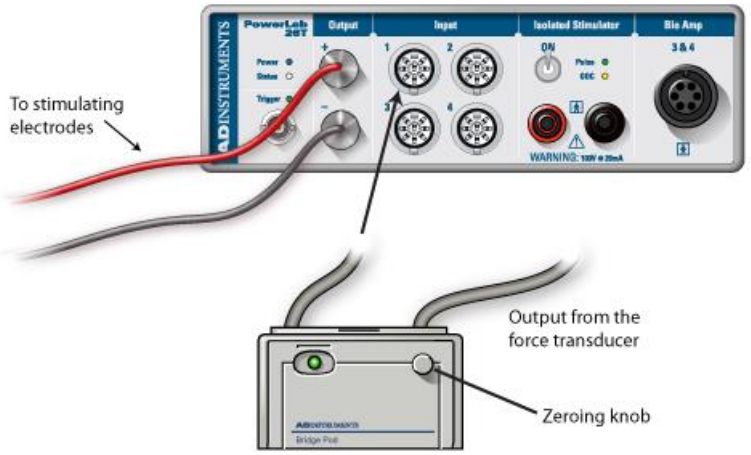


Figure 7. Equipment Setup for PowerLab 26T

3. Securely mount the Force Transducer and Manipulator/Micropositioner on the Ring Stand.
4. Turn on the PowerLab.

Calibrating the Force Transducer

Raw output from the Force Transducer is in millivolts (mV). It needs to be calibrated to give the more meaningful units of Newtons (N). The Force Transducer also has some residual offset voltage that needs to be corrected for.

1. Launch LabChart and open the settings file "Frog Muscle Settings" from the **Experiments** tab in the **Welcome Center**. It will be located in the folder for this experiment. ****_**BE SURE TO LOAD 2 WINDOWS, ONE FOR EACH LIMB****_**
2. Select **Bridge Pod** from the Channel 1 Channel Function pop-up menu. Leave the Force Transducer undisturbed. Observe the signal (Figure 9) in the dialog. Zero this signal by turning the knob on the front of the Bridge Pod. Close the Bridge Pod pop-up menu.

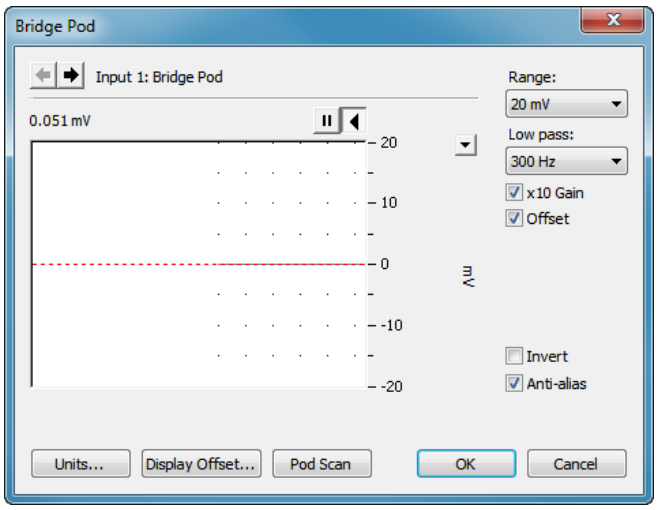


Figure 9. Bridge Pod Dialog

3. **Start** recording. Record for five seconds, and then hang a known weight (between 5–50 grams, provided by your instructor) from the Force Transducer. Record for a further five seconds, and **Stop**.

4. Click-and-drag to **select** all the data. Select **Units Conversion** from the Channel 1 Channel Function pop-up menu (Figure 10).

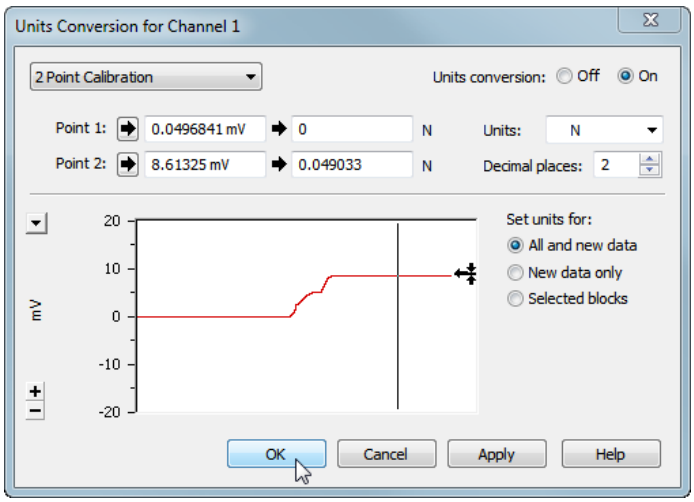


Figure 10. Units Conversion Dialog

5. Select a small area when no weight was added, and click the arrow next to "Point 1."

6. Select a small area when the weight was added, and click the arrow next to "Point 2."

7. Enter the desired unit value in Newtons for each weight. Use the equation below:

$$\text{Force (N)} = \frac{\text{mass (g)}}{1000} \times 9.80665$$

Exercise 1a: Twitch Recruitment

In this exercise, you will determine the effect of stimulus amplitude (strength) on contractile force- the force-intensity relationship.

8. LabChart should be open. If not, open the settings file "Frog Muscle Settings."
9. Make sure the muscle is moist and is in contact with the electrodes.
10. Zero the Bridge Pod. Use the same procedure as before. You do not need to calibrate the data.

Note: For this exercise, you will be running a macro to apply a series of increasing stimuli. A macro is a recorded set of commands and operations that can be executed with a single command.

11. Go to the **Macro** menu and select **Recruitment** to start the macro. Alternatively, you can press **F2**. LabChart will start recording, increase the stimulus on its own, and stop recording. (Do not click Start before playing the macro.)
12. When the macro is finished, select "Save As" and save your data file to the desktop, but do not close the file.
13. Scroll through your data and Autoscale, if necessary. Start at the end of the data and move toward the beginning. Determine the minimum voltage required to elicit the maximum contraction; this is the maximum excitation voltage.
14. Multiply this voltage by 1.5. This new value is the supramaximal stimulus voltage. The supramaximal stimulus voltage will be used in each of the following exercises.
15. Wait at least 30 seconds before moving on to the next exercise. Keep the muscle moist with saline solution.

Exercise 2: Optimizing Muscle Length

In this exercise, you will ensure that muscle length is optimal for force production.

1. Make sure the muscle is moist and the electrodes are still positioned correctly.
2. Zero the Bridge Pod as before. You do not need to calibrate the data.
3. Note the position of the Manipulator/Micropositioner using the ruler.

4. Click on the data at the end of the last data block, and go to the **Commands** menu and select **Add comment**. Type "exercise 2" and click **Add**.

5. Go to the **Setup** menu and select **Stimulator Panel**. In the **Pulse Height** text box, enter the value (in volts) for the supramaximal stimulus voltage calculated in Exercise 1. Do not adjust any other settings.

6. Select **Start** to initiate a single twitch and **Stop** to end the recording

7. Measure the force amplitude with the cursor

8. Lengthen the muscle by 1mm using the micromanipulator and repeat steps 6 & 7.

9. The optimal length has been reached once the force amplitude is unaltered for two successive twitch forces.

Exercise 1b: Twitch Recruitment

In this exercise, you will determine the effect of stimulus amplitude (strength) on contractile force at

optimal length- the force-intensity relationship.

16. LabChart should be open. If not, open the settings file "Frog Muscle Settings."

17. Make sure the muscle is moist and is in contact with the electrodes.

18. Zero the Bridge Pod. Use the same procedure as before. You do not need to calibrate the data.

Note: For this exercise, you will be running a macro to apply a series of increasing stimuli. A macro is a recorded set of commands and operations that can be executed with a single command.

19. Go to the **Macro** menu and select **Recruitment** to start the macro. Alternatively, you can press **F2**. LabChart will start recording, increase the stimulus on its own, and stop recording. (Do not click Start before playing the macro.)

20. When the macro is finished, select "Save As" and save your data file to the desktop, but do not close the file.

21. Scroll through your data and Autoscale, if necessary. Start at the end of the data and move toward the beginning. Determine the minimum voltage required to elicit the maximum contraction; this is the maximum excitation voltage.

22. If the maximal voltage is different than what was found in Exercise 1a, then multiply this voltage by 1.5 and use it throughout the remaining following exercises.

Exercise 3: Muscle Tetanus

In this exercise, you will examine the impact of stimulus frequency on muscle force production- the force-frequency relationship.

1. Make sure the muscle is moist and the electrodes are still positioned correctly.

2. Zero the Bridge Pod as before, but do not calibrate the data.

3. Click on the data at the end of the last data block, and go to the **Commands** menu and select **Add comment**. Type "exercise 2" and click **Add**.

Note: For this exercise, you will be running a different macro to set up the Stimulator and record the data. Do not click Start before playing the macro.

4. Go to the **Macro** menu and select **Tetanus** to start the macro. Alternatively, you can press **F5**. LabChart will start recording and will prompt you with messages.

5. Follow the on-screen instructions. The PowerLab will stimulate the muscle for one second with repetitive pulses at intervals of 400 ms, 200 ms, 100 ms, 50 ms, and then 20 ms. Each recording will appear in a separate block.

6. When the macro has finished, add a comment to each block of data with the stimulus interval as indicated above. Click on the data at the beginning of a data block, and go to the **Commands** menu and select **Add comment**. Type the stimulus interval and select the comment to be inserted at the selection.

7. Save your data. Do not close the file.

8. Wait at least 30 seconds before moving on to the next exercise. Make sure you keep the muscle moist with saline solution.

Exercise 4: Muscle Fatigue

In this exercise, you will examine muscle fatigue, evoked by prolonged tetanic stimulation of the muscle at 50 Hz for 30 seconds.

1. Make sure the muscle is moist and the electrodes are still positioned correctly.

2. Zero the Bridge Pod as before, but do not calibrate the data.

3. Click on the data at the end of the last data block, and go to the **Commands** menu and select **Add comment**. Type "exercise 3" and click **Add**.

Note: For this exercise, you will be running a final macro to stimulate the muscle repetitively. Do not click Start before playing the macro.

4. Go to the **Macro** menu and select **Fatigue** to start the macro. Alternatively, you can press **F6**. LabChart will start recording and will prompt you with messages.

5. Follow the on-screen instructions. The PowerLab will stimulate the muscle with a stimulus interval of 20 ms for 30 seconds. The recording duration of the macro is 45 seconds.

6. Save your data and close the file. **Complete exercises 1-4 with the contralateral limb using the second window you opened**. **If the same settings file/window is used, LabChart will begin the recruitment macro at the last stimulus intensity used- tetanus- which is not appropriate for a twitch.**

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Appendix D: α-SMA Staining Protocol

Materials

24-well culture plates (Cat#: 3738, Corning Incorporated)
PBS
0.1% Saponin (Cat#: 47036, Sigma-Aldrich)
2% Bovine Serum Albumin (Cat# B6917, Sigma Aldrich)
Monoclonal Anti-Alpha Smooth Muscle Actin, Cy3 Conjugate (Cat#: C6198, Sigma-Aldrich)
Slides
Coverslips
Parafilm
Aluminum foil

Staining

1. Using forceps, remove muscle from PBS (stored in microcentrifuge tube at 4°C) and place in a single well of a 24-well culture plate.
2. Prepare antibody solution containing 1:200 1A4 clone (alpha-smooth muscle actin, Cy3 conjugate) in 0.1% saponin (reconstituted in PBS), 2% BSA (reconstituted in PBS) in PBS, using 0.3mL of solution per muscle.
3. Incubate muscle in antibody solution for 3 nights (72 hours) at 4°C). (Note: Critical step—3 nights crucial for bright staining) by gently pipetting solution over muscle.)
4. Wash in 0.1% saponin in PBS 3x for 20 minutes at room temperature. Cover plate with foil during each wash.
5. Wash in plain PBS for 30 minutes. Cover with foil during each wash.
6. Place 1-2 drops of 50/50 PBS and Glycerol onto slide.
7. Remove muscle from well using forceps and place on a slide.
8. Add 1-2 drops of 50/50 PBS and Glycerol to the top of the muscle and place cover slips over the
muscle.
9. Paint edges of coverslip with clear nail polish to create a seal and prevent tissue desiccation.
10. Store slides at 4°C wrapped in foil or an opaque container between imaging.

Imaging

11. Image using a standard fluorescent microscope. (Cy3 excitation: 550 nm, emission: 570 nm)

Appendix E: Gracilis Image Acquisition and Quantification

CAL POLY SAN LUIS OBISPO	BMED 545: Cell Transplantation and Therapeutics	DATE 03/10/15	PAGE 1	OF 1
		WRITER AAS	NO.	REV. 1
TITLE **Gracilis Image Acquisition and Quantification**				

1.0 PURPOSE

This protocol serves as a reference checklist to analyze and quantify the paraformaldehyde fixed and fluorescent antibody stained alpha-smooth muscle actin of mice gracilis muscle under a wide field fluorescence microscope by 1) photographing three regions of collateral (stem, midzone, and reentry) and 2) analyzing their respective diameters using ImageJ's straight line tool.

2.0 Equipment

- Fluorescent stained gracilis muscle slide
- Computer
- Fluorescence Microscope
- ImageJ

3.0 Methods

3.1 Gracilis Image Acquisition

- ☐ Obtain stained muscle slide from ISA.
- ☐ Place under fluorescent microscope.
- ☐ Focus slide through microscope
- ☐ Open computer application.
- ☐ Find profunda end of muscle.
- ☐ Take a picture, rename, and save.
- ☐ Move microscope field right allowing for image overlap on the left side.
- ☐ Take a picture, rename, and save.
- ☐ Repeat above 2 steps until you have reached the far-sided saphenous.
- ☐ Lay stomach down (dorsal)

3.2 Analysis of Gracilis Images

- ☐ Open ImageJ
- ☐ Open scale micrometer
- ☐ Select straight-line tool
  - Draw a line across several graduations
  - Measure line (length) accordingly (in pixels)
- ☐ Select Analyze
  - Set scale
  - Enter length (pixels)
  - Enter known length number
  - Enter units (microns)
- ☐ Use straight-line tool (ctrl+m) to measure 3 regions shown in Figure 1:
  - Collateral stem
  - Collateral midzone
  - Collateral reentery

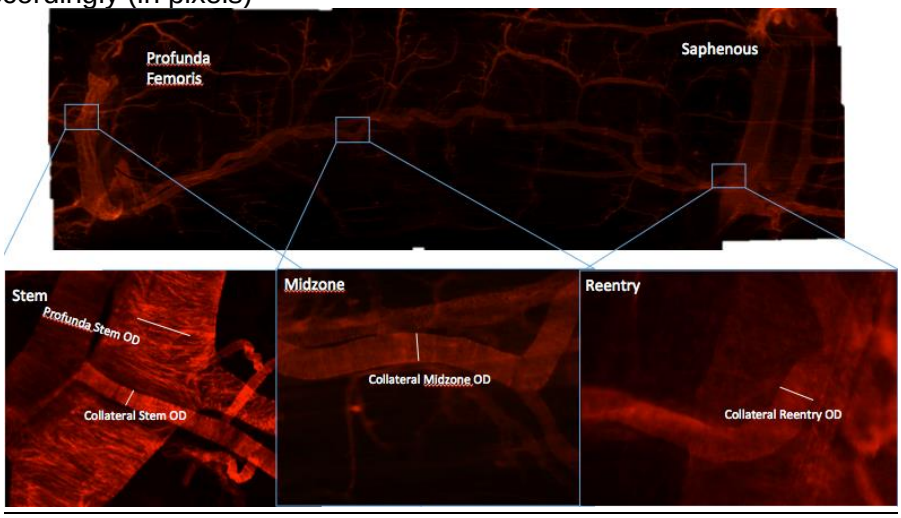


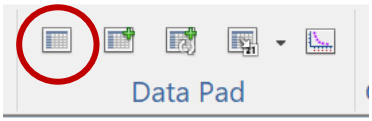
Figure 1: Three regions of gracilis that are to be analyzed: stem, midzone, and reentry.

Appendix F: Data Pad Lab Chart Reader Protocol for Data Acquisition

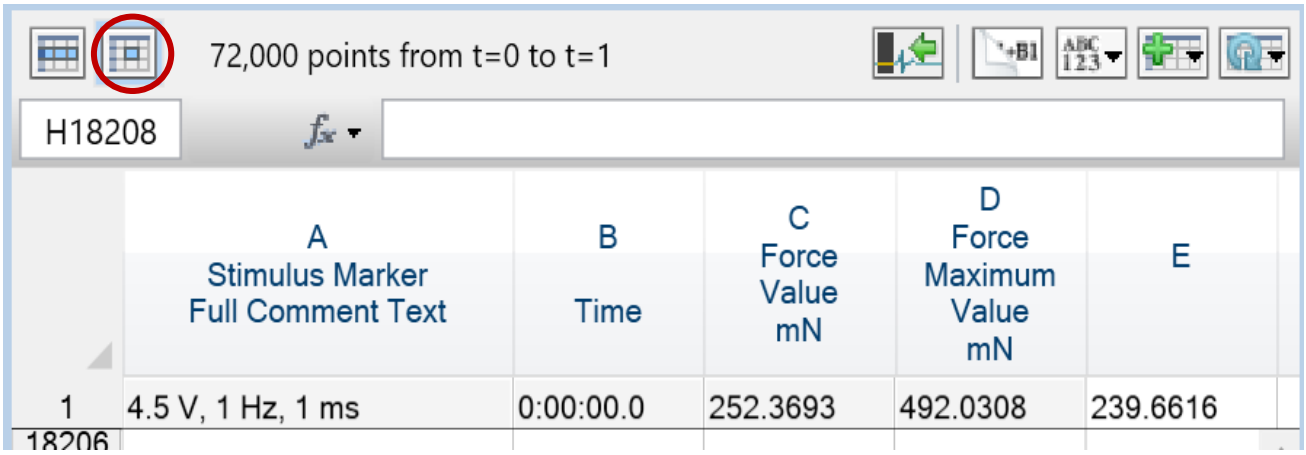
Lab Chart Reader: Data Acquisition Data Pad Protocol

I. Force-Intensity and Force-Frequency

1. Remove "M" marker from chart view.
2. In Data Pad toolbar, select **Data Pad View**



3. Set up needed columns according to **Figure 1**
    a. Click on the heading of Column A
        i. Click *Comments* on left
        ii. Click *Full* Comment Text on right
        iii. Under Calculation Source: select *Stimulus Marker* from drop-down menu
    b. Click on the heading of Column B
        i. Click *Selection & Active Point* on left
        ii. Click *Time* on right
    c. Click on heading of Column C
        i. Click *Selection & Active Point* on left
        ii. Click *Value* on right
        iii. Under Calculation Source: select *Force* from drop-down menu
    d. Click on heading of Column C and D
        i. Click *Statistics* on left
        ii. Click *Maximum Value* on right
        iii. Under Calculation Source: select *Force* from drop-down menu
    e. To find the difference in Force Production
        i. Change Data Pad to **Cell edit mode** in the top left corner of window signified by red circle in **Figure 1**
        ii. Click on the first cell in Column E (cell E1)
        iii. Type "=D1-C1"



	A Stimulus Marker Full Comment Text	B Time	C Force Value mN	D Force Maximum Value mN	E
1	4.5 V, 1 Hz, 1 ms	0:00:00.0	252.3693	492.0308	239.6616
18206					

Figure 1: Relevant Data for Force-Intensity Analysis

4. Return to Chart View Window
    a. Select all data from Exercise 1B as shown in **Figure 2**

        **Note: Be sure to select all data → from 0.0s in 0.25mV to 1.0ms in 50Hz

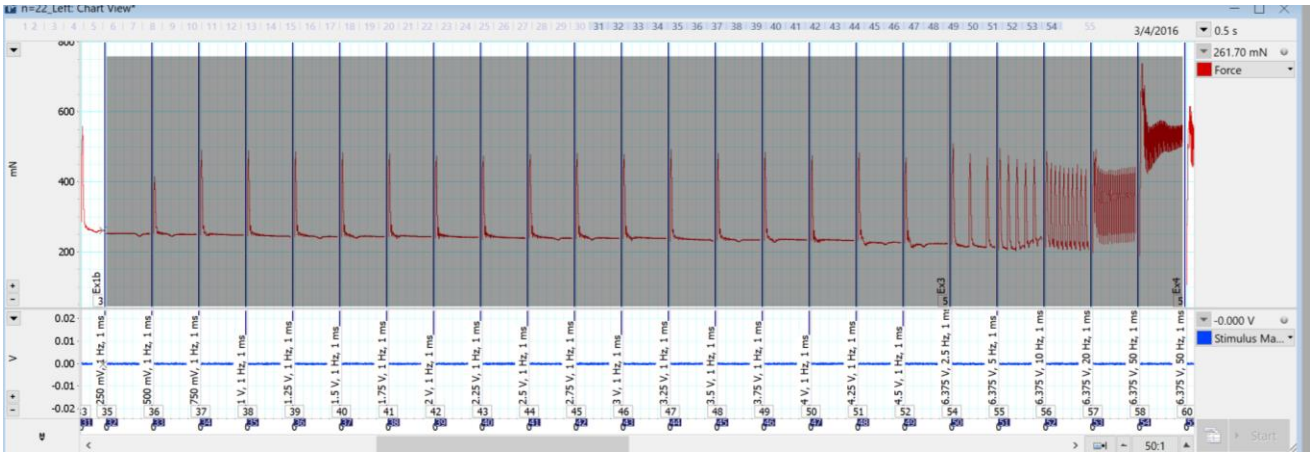
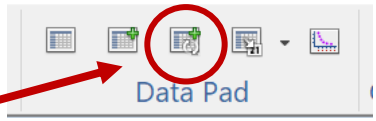
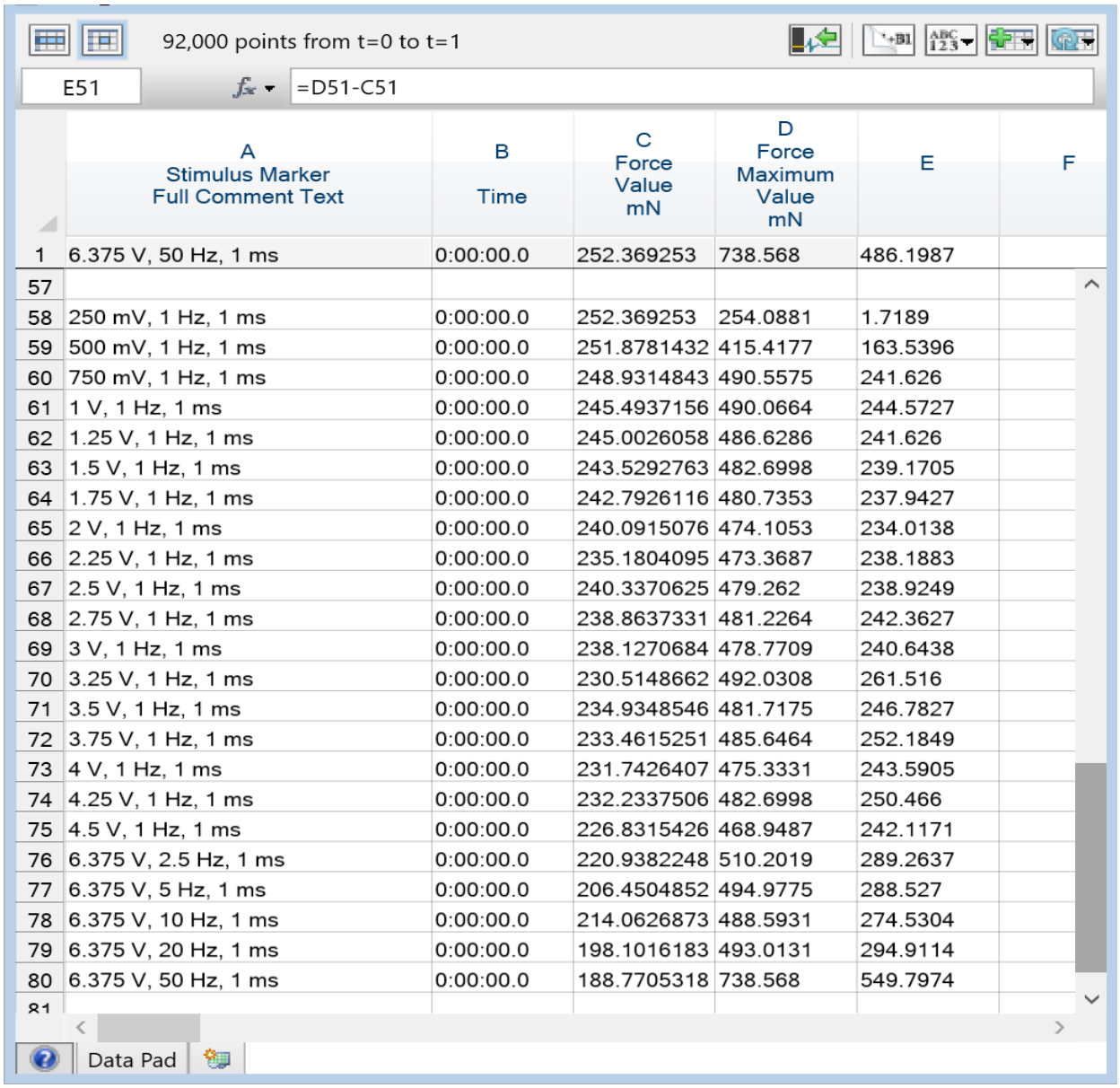


Figure 2: Selection of Exercise 1B in Chart View



5. To populate Data Pad
    a. Select **Multiple Add to Data Pad** in Data Pad toolbar
        i. In *Select* field input "1.0" s"
        ii. For Step Through select *Current Selection*
        iii. Click Add button.
6. Data values can then be copied and pasted into Excel



92,000 points from t=0 to t=1

E51 =D51-C51

	A Stimulus Marker Full Comment Text	B Time	C Force Value mN	D Force Maximum Value mN	E	F
1	6.375 V, 50 Hz, 1 ms	0:00:00.0	252.369253	738.568	486.1987	
57						
58	250 mV, 1 Hz, 1 ms	0:00:00.0	252.369253	254.0881	1.7189	
59	500 mV, 1 Hz, 1 ms	0:00:00.0	251.8781432	415.4177	163.5396	
60	750 mV, 1 Hz, 1 ms	0:00:00.0	248.9314843	490.5575	241.626	
61	1 V, 1 Hz, 1 ms	0:00:00.0	245.4937156	490.0664	244.5727	
62	1.25 V, 1 Hz, 1 ms	0:00:00.0	245.0026058	486.6286	241.626	
63	1.5 V, 1 Hz, 1 ms	0:00:00.0	243.5292763	482.6998	239.1705	
64	1.75 V, 1 Hz, 1 ms	0:00:00.0	242.7926116	480.7353	237.9427	
65	2 V, 1 Hz, 1 ms	0:00:00.0	240.0915076	474.1053	234.0138	
66	2.25 V, 1 Hz, 1 ms	0:00:00.0	235.1804095	473.3687	238.1883	
67	2.5 V, 1 Hz, 1 ms	0:00:00.0	240.3370625	479.262	238.9249	
68	2.75 V, 1 Hz, 1 ms	0:00:00.0	238.8637331	481.2264	242.3627	
69	3 V, 1 Hz, 1 ms	0:00:00.0	238.1270684	478.7709	240.6438	
70	3.25 V, 1 Hz, 1 ms	0:00:00.0	230.5148662	492.0308	261.516	
71	3.5 V, 1 Hz, 1 ms	0:00:00.0	234.9348546	481.7175	246.7827	
72	3.75 V, 1 Hz, 1 ms	0:00:00.0	233.4615251	485.6464	252.1849	
73	4 V, 1 Hz, 1 ms	0:00:00.0	231.7426407	475.3331	243.5905	
74	4.25 V, 1 Hz, 1 ms	0:00:00.0	232.2337506	482.6998	250.466	
75	4.5 V, 1 Hz, 1 ms	0:00:00.0	226.8315426	468.9487	242.1171	
76	6.375 V, 2.5 Hz, 1 ms	0:00:00.0	220.9382248	510.2019	289.2637	
77	6.375 V, 5 Hz, 1 ms	0:00:00.0	206.4504852	494.9775	288.527	
78	6.375 V, 10 Hz, 1 ms	0:00:00.0	214.0626873	488.5931	274.5304	
79	6.375 V, 20 Hz, 1 ms	0:00:00.0	198.1016183	493.0131	294.9114	
80	6.375 V, 50 Hz, 1 ms	0:00:00.0	188.7705318	738.568	549.7974	
81						

Data Pad

Figure 3: Example Data Pad Output for Force-Intensity and -Frequency

II. Fatigue

1. Place “M” marker at 0.00s in Fatigue window
2. Click anywhere on fatigue curve to set sample parameters
3. In *Data Pad* toolbar, select **Data Pad View**
4. Set up needed columns according to **Figure 4**
    a. Click on Column G heading

i. Click *Selection & Active Point* on left

ii. Click Time on *Time* on right

b. Column H to J

i. Click *Statistics* on left

ii. Click the relevant measurement for each column on the right

iii. Under Calculation Source: select *Force* from drop-down menu

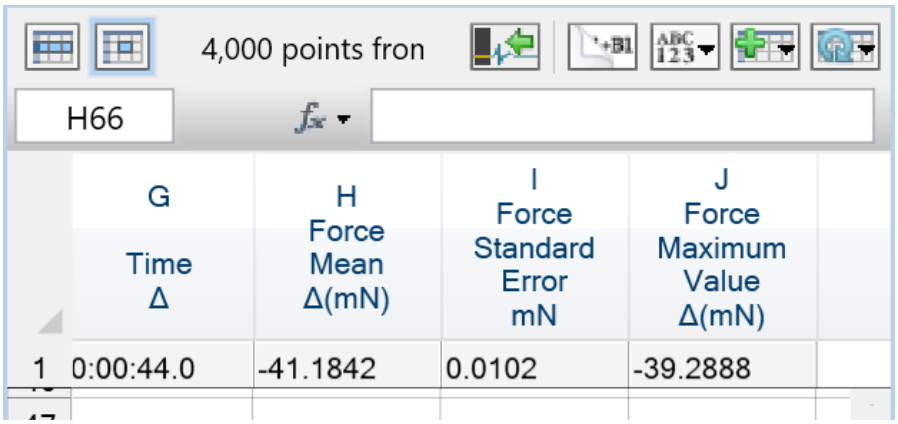


Figure 4: Relevant Data Needed for Fatigue Analysis

5. Select **Multiple Add to Data Pad** in Data Pad toolbar

   a. In *Select* field input "1.0" s"

   b. For Step Through select *Block containing selection*

   c. Click Add button.

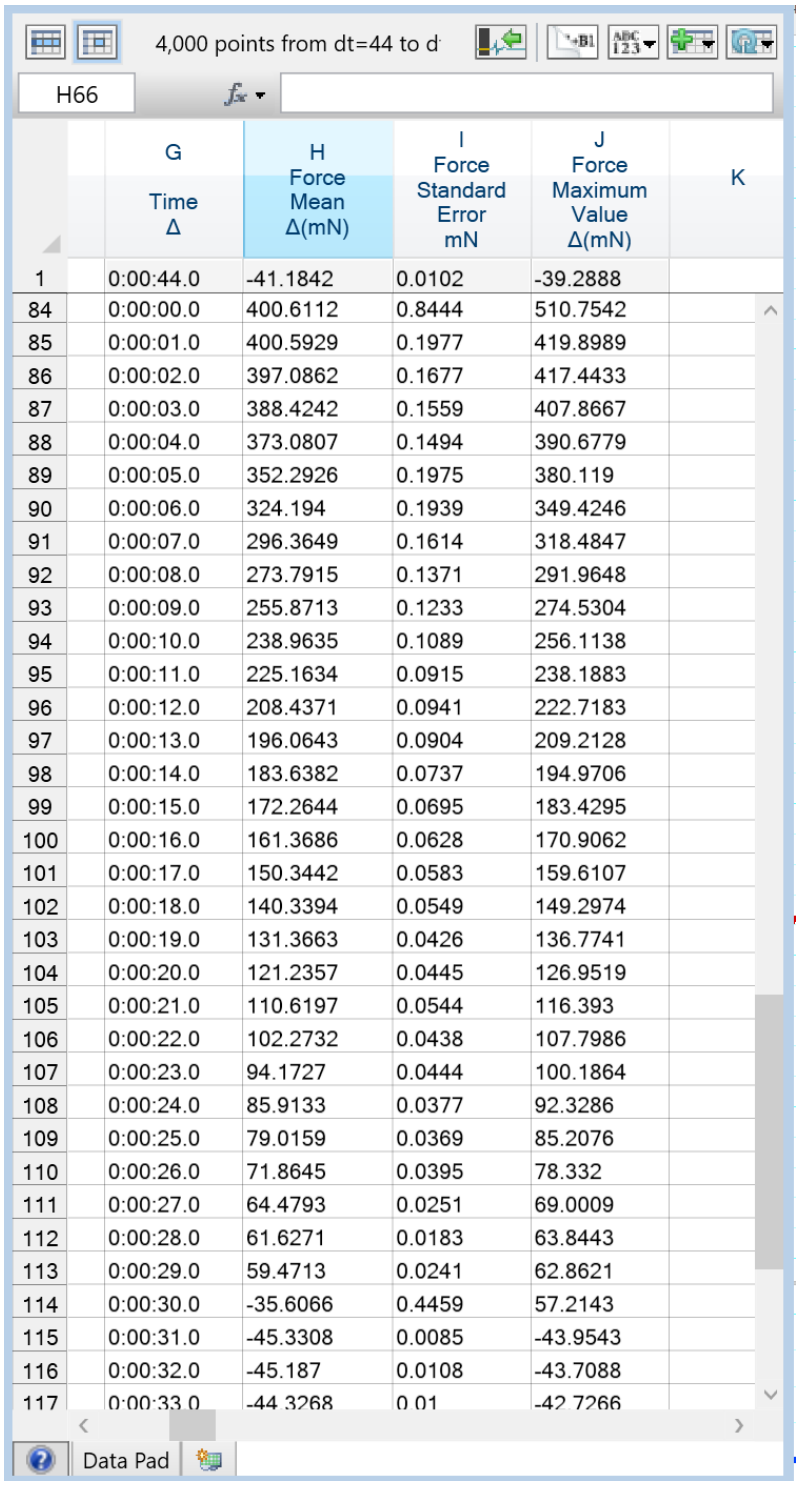
6. Inactivate all other headings.

   a. Click on column heading.

   b. Select "Off".

7. Data values can then be copied and pasted into Excel

   **Note: use "Max Force Value" at 0.00s as 100% value when calculating percentages



	G Time Δ	H Force Mean Δ(mN)	I Force Standard Error mN	J Force Maximum Value Δ(mN)	K
1	0:00:44.0	-41.1842	0.0102	-39.2888	
84	0:00:00.0	400.6112	0.8444	510.7542	
85	0:00:01.0	400.5929	0.1977	419.8989	
86	0:00:02.0	397.0862	0.1677	417.4433	
87	0:00:03.0	388.4242	0.1559	407.8667	
88	0:00:04.0	373.0807	0.1494	390.6779	
89	0:00:05.0	352.2926	0.1975	380.119	
90	0:00:06.0	324.194	0.1939	349.4246	
91	0:00:07.0	296.3649	0.1614	318.4847	
92	0:00:08.0	273.7915	0.1371	291.9648	
93	0:00:09.0	255.8713	0.1233	274.5304	
94	0:00:10.0	238.9635	0.1089	256.1138	
95	0:00:11.0	225.1634	0.0915	238.1883	
96	0:00:12.0	208.4371	0.0941	222.7183	
97	0:00:13.0	196.0643	0.0904	209.2128	
98	0:00:14.0	183.6382	0.0737	194.9706	
99	0:00:15.0	172.2644	0.0695	183.4295	
100	0:00:16.0	161.3686	0.0628	170.9062	
101	0:00:17.0	150.3442	0.0583	159.6107	
102	0:00:18.0	140.3394	0.0549	149.2974	
103	0:00:19.0	131.3663	0.0426	136.7741	
104	0:00:20.0	121.2357	0.0445	126.9519	
105	0:00:21.0	110.6197	0.0544	116.393	
106	0:00:22.0	102.2732	0.0438	107.7986	
107	0:00:23.0	94.1727	0.0444	100.1864	
108	0:00:24.0	85.9133	0.0377	92.3286	
109	0:00:25.0	79.0159	0.0369	85.2076	
110	0:00:26.0	71.8645	0.0395	78.332	
111	0:00:27.0	64.4793	0.0251	69.0009	
112	0:00:28.0	61.6271	0.0183	63.8443	
113	0:00:29.0	59.4713	0.0241	62.8621	
114	0:00:30.0	-35.6066	0.4459	57.2143	
115	0:00:31.0	-45.3308	0.0085	-43.9543	
116	0:00:32.0	-45.187	0.0108	-43.7088	
117	0:00:33.0	-44.3268	0.01	-42.7266	

Figure 5: Example Data Pad Output for Fatigue

Appendix G: Relevant Statistical Analysis for Force-Intensity Production Improvements

General Linear Model: Force versus Interval, Group

```
Method

Factor coding (-1, 0, +1)


Factor Information

Factor  Type  Levels Values
Interval Fixed   18 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75,
             3.00, 3.25, 3.50, 3.75, 4.00, 4.25, 4.50
Group   Fixed    4 Cells, Collagen, Control, Pocket


Analysis of Variance

Source       DF  Adj SS  Adj MS F-Value P-Value
 Interval     17  29.323  1.7249   5.96  0.000
 Group         3  33.471 11.1571  38.58  0.000
 Interval*Group  51  1.979  0.0388   0.13  1.000
Error       432 124.937  0.2892
Total       503 203.116


Model Summary

    S  R-sq R-sq(adj) R-sq(pred)
0.537779 38.49%   28.38%   12.66%
```

Fisher Pairwise Comparisons: Response = Force, Term = Interval

```
Grouping Information Using Fisher LSD Method and 95% Confidence

Interval  N   Mean   Grouping
4.50   28 1.07992 A
4.25   28 1.05335 A
3.75   28 1.04894 A B
4.00   28 1.04599 A B
3.50   28 1.02427 A B
3.25   28 1.00178 A B
3.00   28 0.97665 A B
2.75   28 0.93990 A B C
2.50   28 0.91738 A B C
2.25   28 0.86465 A B C
2.00   28 0.82123 A B C D
1.75   28 0.81362 A B C D
1.50   28 0.78538 A B C D
1.25   28 0.73183   B C D
1.00   28 0.64969    C D
0.75   28 0.53074      D E
0.50   28 0.28455       E F
0.25   28 0.06904         F

Means that do not share a letter are significantly different.
* NOTE * Cannot draw the interval plot for the Fisher procedure. Interval plots for
     comparisons are illegible with more than 45 intervals.
```