

Development and Characterization of PLGA and ePTFE Blood Vessel Mimics Using Gene Expression Analysis

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CHAPTER 1: Introduction

Blood Vessel Mimics

Overview

Coronary artery disease (CAD) affects millions of people, causing approximately one death in the U.S. every minute¹. The use of small intravascular devices like stents is one potential way to treat CAD. However, current stent testing procedures are inefficient at best. The blood vessel mimic (BVM) has been created to address this issue, utilizing concepts from tissue engineering to create a more complex in vitro model for stent testing². Current evaluation techniques for these polymer-cell constructs include a wide variety of stains and histology as well as fluorescence and scanning electron microscopy. The two goals of this project were to improve the protocol for creating a BVM on expanded polytetrafluoroethylene (ePTFE) and Poly Lactic-co-Glycolic Acid (PLGA) using human umbilical vein endothelial cells (HUVEC's) and smooth muscle cells (HUVSMC's), and to develop a protocol for evaluating gene expression in the BVM's produced using real-time, reverse-transcription polymerase chain reaction (RT-PCR). Therefore, to provide a foundation for the work, background will be provided on coronary artery disease, stenting and current stent testing methods, the need for an improved model for testing, and the theory behind the composition of the BVM.

Coronary Artery Disease

According to the American Heart Association, cardiovascular disease accounts for one of every 2.9 deaths in the U.S., with coronary artery disease alone accounting for one of every six deaths¹. CAD involves a narrowing of the coronary arteries caused by the accumulation of lipid-based plaques on the walls of the vessels through a process known as atherosclerosis³(Figure 1).

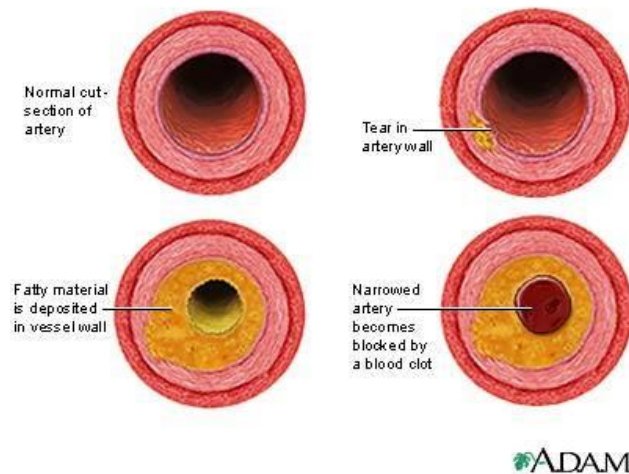


Figure 1. Initiation and development of atherosclerotic plaque

The plaques, composed mainly of fibrous tissue, calcified tissue, and amorphous debris containing cholesterol and various forms of extracellular lipids⁴, form as a result of injury or damage to the endothelium of the vessel(Figure 2). Certain risk factors, such as age, family history, gender (male), high blood pressure, high cholesterol, diabetes, smoking, and many others increase the likelihood of a patient developing CAD³.

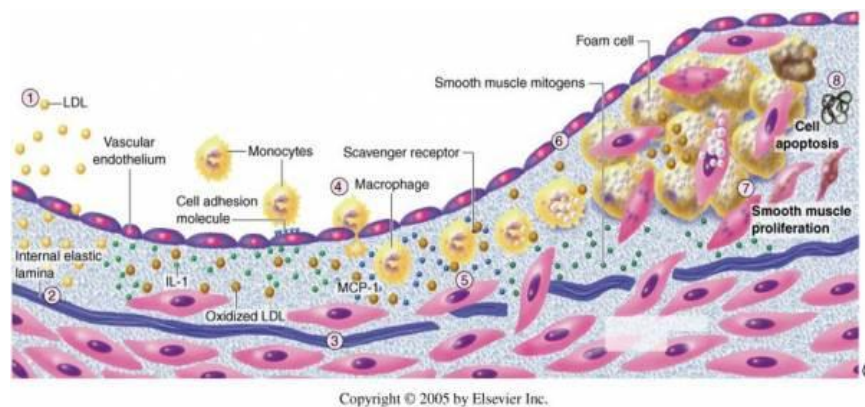


Figure 2. Cross-sectional view of atherosclerotic plaque formation

The narrowing of the arteries slowly decreases blood flow to the heart muscle, which generally leads to angina (chest pain), shortness of breath, fatigue and weakness. The narrowing and hardening of the arteries that result from atherosclerosis leads to an increased incidence of blood clotting at the site of the plaque as well as an increased susceptibility to vessel occlusion, a potentially fatal combination that often leads to myocardial infarction, or heart attack (Figure 3). Treatments for CAD range from balloon angioplasty and stenting to coronary artery bypass grafting (CABG)⁵.

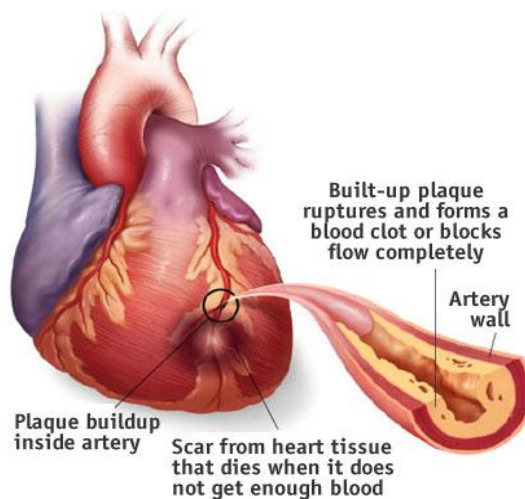


Figure 3. Buildup of atherosclerotic plaque leading to myocardial infarction and cardiac tissue death

Stenting

In 1986, Sigwart and Puel were the first to implant stents into human coronary arteries⁶. Stents are devices that are deployed in the lumen of a diseased vessel to provide mechanical support to the vessel and ensure that it remains patent, or open. Before the development and implementation of stents, a procedure known as balloon angioplasty was developed in which a balloon catheter was deployed within the stenotic vessel to compress the walls of the vessel outward and reopen the lumen to restore flow. Stents were developed to address the issues of restenosis and occlusion

that often result after balloon angioplasty; because stenting does not treat or remove the diseased tissue, restenosis in these patients occurs at the alarming rate of 30 to 40 percent⁷. By creating a permanent (or at least a long-term) device that will mechanically hold the vessel open after balloon angioplasty, it is possible to substantially increase both short and long-term patency and patient survival as compared to balloon angioplasty alone⁸(Figure 4).

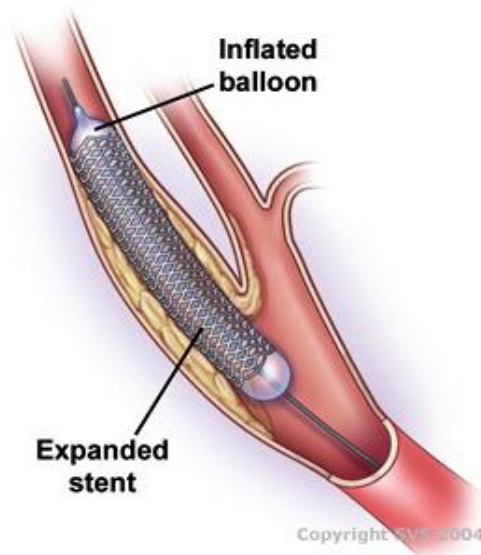


Figure 4. The stent is guided into the artery via a catheter, where upon reaching the target location the balloon is inflated and the stent is deployed.

The first stents, developed by Palmaz and Schatz, were bare metal scaffolds that only performed a mechanical function⁹. While they did improve patency and reduce restenosis, they also created new issues, such as thrombus (blood clot) formation on the exposed metal and potentially hemolytic flow conditions near the device. As the field has progressed, new materials and modifications have continued to improve overall stent function, even when the new variations come with their own unique set of concerns. The first Palmaz-Schatz stents were unmodified

stainless steel(Figure 5); now stents are made of a variety of other materials as well, including cobalt-chromium alloys¹⁰ and nickel-titanium alloys (i.e. NiTiNOL)¹¹.

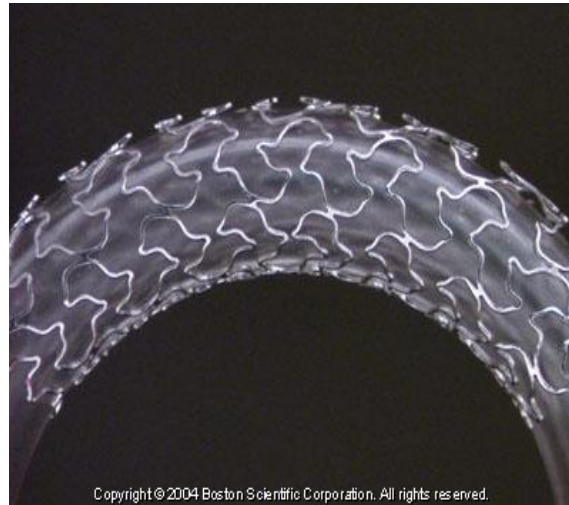


Figure 5. The Veriflex bare metal stent from Boston Scientific represents the latest in bare metal stainless steel stent design

These base metals can be modified in variety of ways, with the most common modifications involving coatings like paclitaxel, sirolimus, and heparin that aim to reduce thrombogenesis or restenosis. These drug (or in the case of heparin, biomolecular) coatings can either be applied directly to the metal scaffold¹² or as part of a degradable polymer coating on the metal that will slowly release the drug over time¹³. As more materials and potential modifications are discovered, it will be imperative to identify the most promising technologies at an early stage of development in order to ensure that time and resources are spent developing the most effective devices possible.

Current Stent Testing

The current stent testing paradigm involves a series of bench top tests for biocompatibility as well as mechanical properties followed directly by animal testing (Figure 6). The Food and Drug Administration (FDA) has a Guidance Document that outlines recommended tests for

biocompatibility and mechanical testing of stents¹⁴. The biocompatibility testing is mediated almost entirely by the International Organization for Standardization document 10993 (ISO 10993), a comprehensive set of twenty evaluation techniques designed to thoroughly test medical devices before their use in humans, while the mechanical testing sections look to simulate physical forces that might be experienced by the stent in vivo. While these evaluation procedures do an adequate job in identifying areas of concern in stent composition and mechanical function, the leap from simple, in vitro analysis into animal models leads to a great deal of time and resources wasted on designs that seem to work in simplified testing conditions but fail when they are exposed to the complex environment of native vasculature.

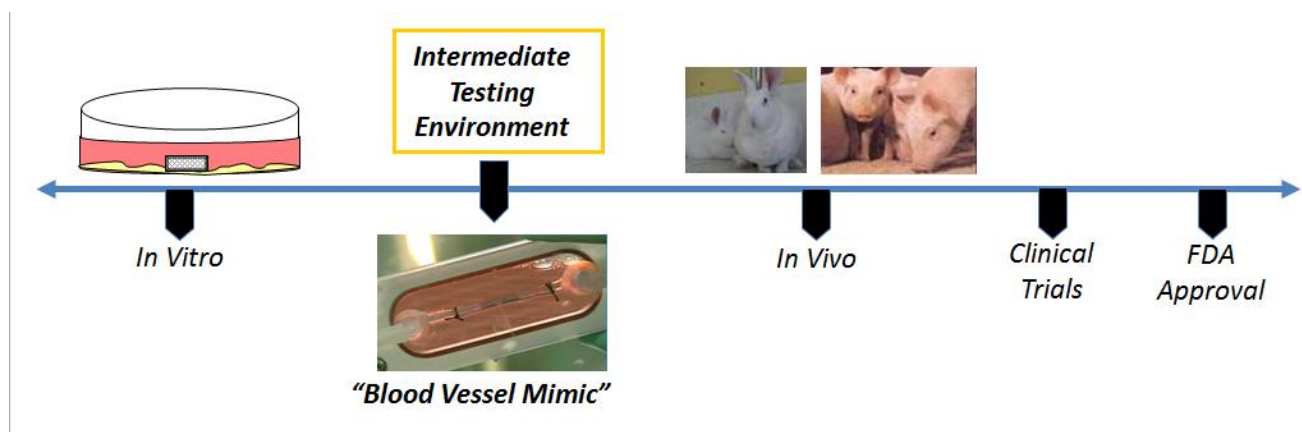


Figure 6: A Diagram of the Current Stent Testing Paradigm

Need for Improved In Vitro Model

A more physiologically accurate in vitro model of native vasculature has the potential to ameliorate both the high costs and ethical concerns of high throughput animal testing. This is the theory behind the blood vessel mimic (BVM), which applies the tissue engineering approach of seeding human cells on a tubular scaffold, in this case a polymer, to create a tissue engineered vascular graft (TEVG) that can be used for testing intravascular devices like stents in a three-dimensional, physiologically relevant setting². The BVM creates an intermediate testing step

between the bench top and animal models that allows for stent testing in a dynamic, physiologic and, most importantly, controlled environment at much lower costs than the animal models it can replace. Of course, the BVM will not eliminate all animal testing, as the in vitro environment at this point cannot fully simulate the complexity of native vessels in living organism. The goal of the BVM is to replace some animal models and serve as a high throughput testing method to help identify the strongest candidates for further testing in animal models.

Native Vasculature

In order to create a relevant in vitro model it is important to begin with an understanding of the native vasculature that we are attempting to model. In general, arteries are tubular structures composed of an innermost endothelial cell intima surrounded by a smooth muscle media, all surrounded by the loose connective tissue of the adventitia¹⁵(Figure 7).

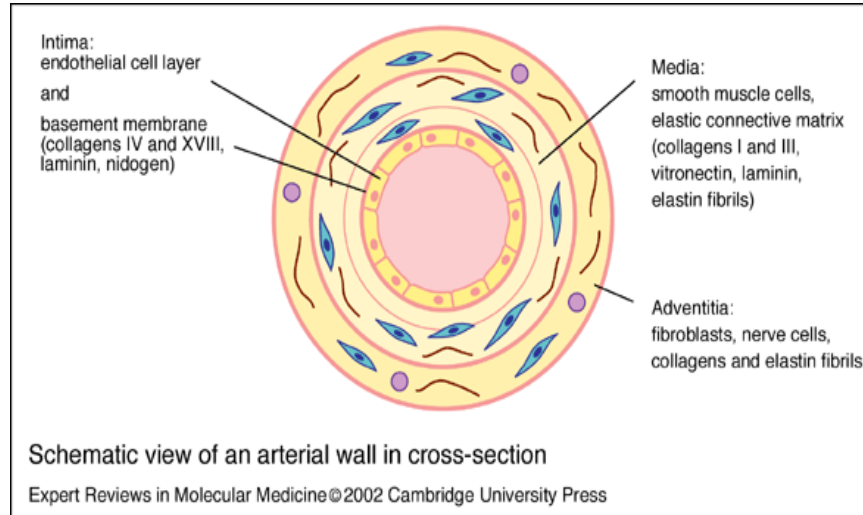


Figure 7. Cross-sectional view demonstrating the three key layers of the native artery.

The key cell types of each layer include endothelial cells (EC's) in the blood contacting intima, smooth muscle cells (SMC's) in the media, and fibroblasts in the adventitia.

Endothelial Cells

Endothelial cells are the primary component of the endothelium, or tunica intima, the cell layer that directly contacts the blood and acts as an anti-coagulant layer. Endothelial cells also form tight junctions, and allow for selective transport of materials into or out of the bloodstream¹⁶. During inflammation, a damaged endothelium will express cell adhesion molecules such as intercellular adhesion molecule (ICAM) to allow for the extravasation of leukocytes into the interstitium¹⁷. Endothelial cells can also secrete factors that can stimulate smooth muscle cells to either contract or relax, which in turn either increases or decreases blood pressure and flow¹⁸.

Smooth Muscle Cells

The tunica media is made up of smooth muscle cells, collagen, and other elastic proteins and is the thickest of all the layers of an artery. Located outside of the tunica intima, the tunica media is responsible for contraction or relaxation of vessel diameter¹⁸. There is a complex interplay between endothelial cells and smooth muscle cells, involving the connection of vasodilators, factors that cause vessel dilation, and vasoconstrictors, factors that cause vessel constriction, released from the endothelium and sent to the smooth muscle¹⁷. Although some of these pathways have been explored, such as the role of endothelial cell Calcium levels¹⁹, more characterization of endothelial and smooth muscle cell interaction is necessary.

Extracellular Matrix

The extracellular matrix, and particularly the thin lamina that separate the three layers of the vessel, contain a high concentration of elastic proteins that give the vessel the ability to relax and contract in order to control blood flow in response to signals from the sympathetic and parasympathetic nervous system. The coronary arteries(Figure 8), which range from about 1-6 mm in diameter²⁰, perform the unique function of transporting oxygenated blood to the heart muscle. If blood flow in the coronary arteries is interrupted for even a short amount of time, the

heart muscle downstream of the occluded vessel will die in a process known as myocardial infarction (MI), better known as a heart attack. If the area downstream of the occlusion is large enough, the heart will lose its ability to pump blood to the body and the patient will die.

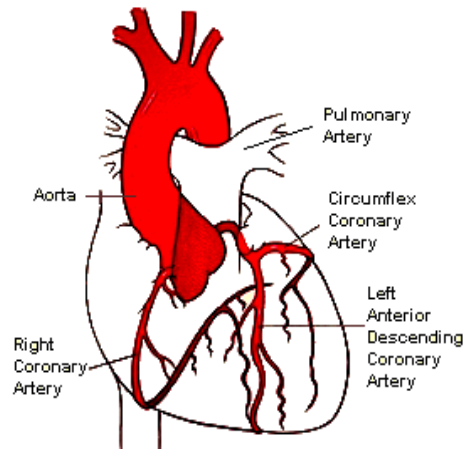


Figure 8. Schematic of the coronary arteries, shown in red.

Tissue Engineering

Tissue engineering traditionally combines cells on a scaffold cultivated in a bioreactor to recreate a complex tissue found in the body. The primary focus of tissue engineering of blood vessels is for the implantation into human with CAD²¹. Cell source is critical for the creation of a TEBV, especially if it is intended to be implanted in a human, then the immune response, the body's natural reaction to foreign cells or material, must be taken into account²¹. However, if the TEBV is not created with the intention of implantation, cell source options increase tremendously, as do scaffold options. The scaffold is another crucial part of tissue engineering, and as such must be able to promote cell adhesion and proliferation, as well as provide the structure for the vessel²¹.

Cell Sources

HUVECs

Endothelial cells vary largely depending upon where in the body they are located, and what type of tissue they are found in²². Human Umbilical Vein Endothelial Cells (HUVECs) are commercially available at a relatively low cost endothelial cell types, as compared to Human Coronary Artery Endothelial Cells (HCAECs). Although HUVECs are not as physiologically relevant as HCAECs to the coronary artery, they are from a large vessel source, which suggests that they will have at least a similar function to HCAECs. HUVECs are also a well characterized cell type, and studies have been performed to determine their base gene expression²³.

HUVSMCs

Much like HUVECS, Human Umbilical Vein Smooth Muscle Cells (HUVSMCs) are commercially available at a relatively low cost. Smooth muscle cells are located in the tunica media¹⁸ and therefore, HUVSMCs would be used to model that layer of the blood vessel. With a successful seeding, the HUVSMCs will arrange in a thick layer outside of the HUVECs, facilitating interactions between the two cell types.

Scaffold Materials

Expanded Polytetrafluoroethylene

Expanded polytetrafluoroethylene (ePTFE), commonly known as Gore-Tex, is a potential scaffold material for BVM construction. From a materials perspective, there are few if any materials with a longer history in vascular applications than ePTFE(Figure 9).



Figure 9. ePTFE sample demonstrating the flexibility of the material

The two major applications of ePTFE are its use in arteriovenous (AV) shunts for hemodialysis²⁴ and permanent vascular grafts in those situations where autologous grafting is not feasible due either to availability or viability of the native vasculature, which is estimated to occur in 30% of patients requiring small-diameter (< 6mm) vascular grafts²⁵. Even though ePTFE is far less compliant than native vasculature²⁶, its porosity, high biocompatibility and hemocompatibility allow it to function reasonably well as a vascular graft material, particularly in large-diameter applications.

The same qualities of biocompatibility and porosity that make ePTFE a quality material for permanent vascular grafts also make it a viable material for a BVM scaffold. Though unmodified ePTFE demonstrates only moderate cell adhesion, many possible modifications to improve cell adhesion are available²⁷⁻³⁰, and the continued survival of cells on ePTFE in vitro is well documented². Because the purpose of the BVM is primarily to analyze endothelial (and possibly medial smooth muscle) reaction to stent implantation, and because stented arteries are always less compliant than normal, healthy vasculature, the high compliance of ePTFE should not negatively impact the validity of the BVM model significantly.

Poly-Lactic-co-Glycolic Acid

Poly Lactide-co- Glycolide Acid (PLGA) is a low cost polymer, and when electrospun provides a nanofibrous tubular scaffold used as the main structural component of a BVM³¹. PLGA was

chosen based on evidence of adequate cell attachment, mechanical properties comparable to native vessels, and biocompatibility³². The nanofibrous structure of the electrospun PLGA mimics the fibrous qualities of the native extracellular matrix, which promotes cell adhesion to the scaffold³³ (Figure 10).

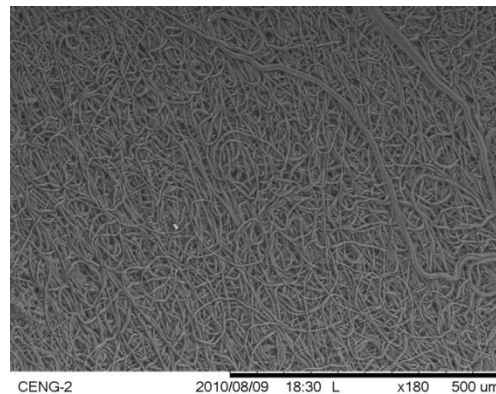


Figure 10: Scanning Electron Microscope image of nanofibrous electrospun PLGA

Gene Expression

Overview

Gene expression, and more specifically Polymerase chain reaction (PCR) is a way to determine the functionality of a tissue or cells by determining the relative amount of mRNA (messenger RiboNucleic Acid) expressed in the tissue. The mRNA correlates to functionality by indicating what proteins are going to be translated from that mRNA, the proteins a tissue is expressing contribute to the function of the cell. The four most common types of methods for assessing gene expression are northern blotting, serial analysis of gene expression, gene chip microarrays, and real-time quantitative PCR (qPCR)³⁴. There are three main steps that go into determining gene expression in a given tissue: RNA isolation, cDNA synthesis, and qPCR.

Gene Expression in the BVM

Isolation of mRNA from tissues is the first step in the qPCR process. mRNA is isolated by lysing the sample, or rupturing the cell membrane, to release the cell contents, and the lysate is then

processed through a series of filtrations to separate the mRNA from other cellular components. This mRNA must then be reverse transcribed into cDNA (complementary deoxyribonucleic acid). Next, the cDNA goes through a cycle of dissociation and annealing steps, to first separate the strands of DNA from each other and then annealing to combine both a probe and mRNA sequence-specific primers to bind to a single DNA strand. The mRNA specific primers are roughly 20 base pairs long and are specific to both the forward and reverse strand of a specific cDNA gene copy. After the primers have attached, polymerase enzymes use free nucleotides in solution to reconstruct the complimentary strand one base at a time. The amount of DNA present is directly measured by a fluorescent reporter that is activated by binding to double stranded DNA. A real-time PCR machine records the fluorescence after each cycle, and from there a threshold cycle can be calculated. Threshold cycle data can then be normalized to a reference gene, a gene that has a constant expression rate independent of the fluctuations of the other genes in the cell, and processed through a series of equations to determine relative expression amounts¹⁷. A schematic of the general steps of qPCR can be seen in Figure 11 below.

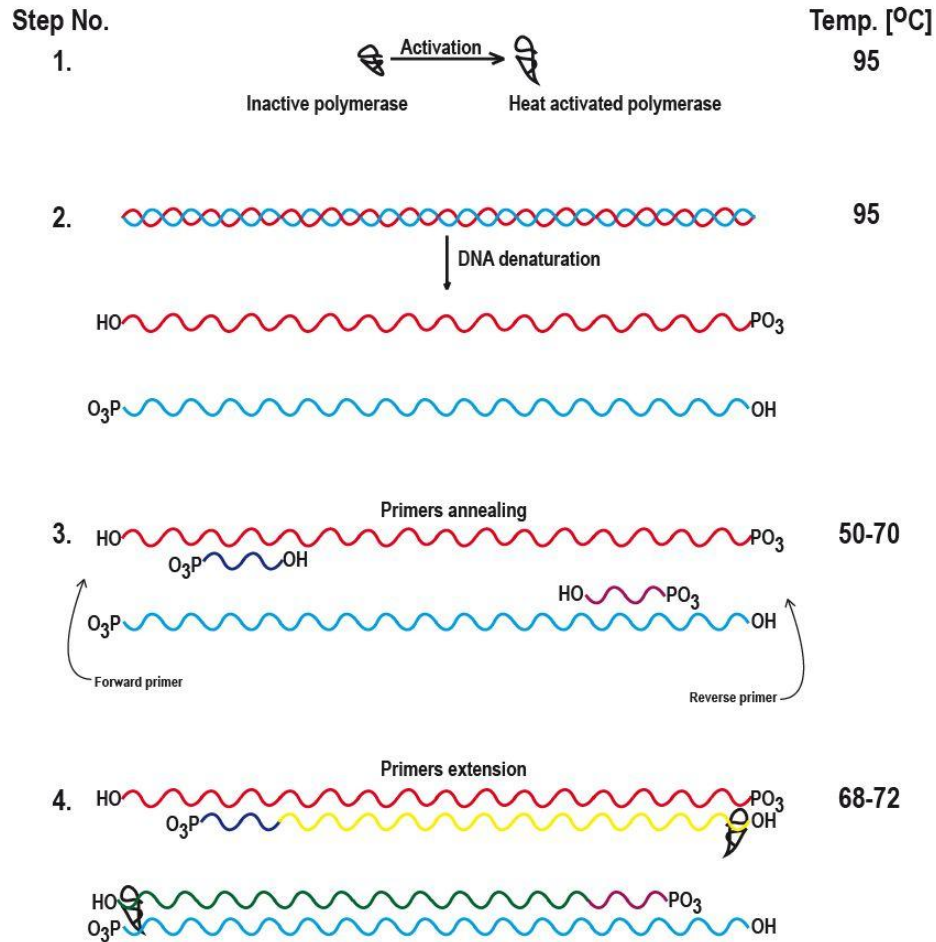


Figure 11: Schematic of qPCR steps³⁵

Overall Goals

There are three major goals that this project aims to accomplish: develop a protocol for PLGA as a scaffold for the BVM system, develop a protocol for ePTFE that results in a vessel that can be used for gene expression, and develop a protocol for quantitative RT-PCR gene expression analysis of both the ePTFE and PLGA vessels.

CHAPTER 2: Methods

BVM Cultivation

This project had two main components, BVM development and gene expression protocol development. This chapter will focus on the methods of both BVM cultivations and gene expression, excluding the results associated with both to provide a more comprehensive view of the project results, which can be found in chapter 3 of this report.

Cell Culture

Human umbilical vein endothelial cells (HUVEC's) and human umbilical vein smooth muscles cells (HUVSMC's) were cultured under standard conditions (37°C, 5% CO₂) in endothelial cell growth media (Lonza EGM-2, Catalog No. cc3162) and smooth muscle cell media (Lonza SmGM-2, Catalog No. cc3182), respectively. HUVEC's were trypsinized and passed at approximately 80% confluence at ratios of either 1:2 or 1:3 to ensure growth and proliferation, as HUVEC's are neighbor-dependent cells that do not grow well when underconfluent³⁶. HUVSMC's were passed at similar confluence and ratios, and though they are less neighbor-dependent high culture density does improve cell proliferation³⁷. All cell culture procedures were carried out in a biological safety cabinet using aseptic techniques.

Bioreactors

The bioreactors used for BVM cultivation consisted of an air tight, locking lid Tupperware container, with an inlet and outlet port (connected by the construct) as well as a release port for use during priming and pressure sodding of the cells (Figure 12). The Tupperware chamber was filled with Bioreactor Media (500mL M199, 56mL FBS, 5mL L-glutamine, 5mL Penicillin/Streptomycin, 0.6mL Fungizone, 2.8mL HEPES) before the scaffold was introduced into the system. The chamber was connected to a media reservoir filled with cell-specific media (either HUVEC media or a combination of HUVEC and HUVSMC for dual-sodded constructs)

via a combination of gas-permeable tubing and pump tubing. A three-way, Luer-Lock inlet (proximal) stopcock allowed syringe attachment for cell delivery, and a two-way outlet (distal) stopcock allowed for the luminal blockage necessary for pressure sodding. A third two-way stopcock downstream of both the outlet and release port allowed the system to be sealed for transportation from the hood to the incubator without spills or contamination. Closed systems can then be mounted onto an 8-roller Masterflex pump in the incubator and conditioned accordingly (Figure 13).

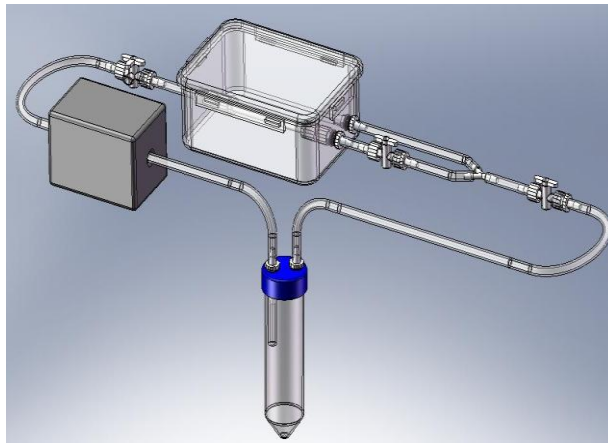


Figure 12: Computer drawing of the Bioreactor components

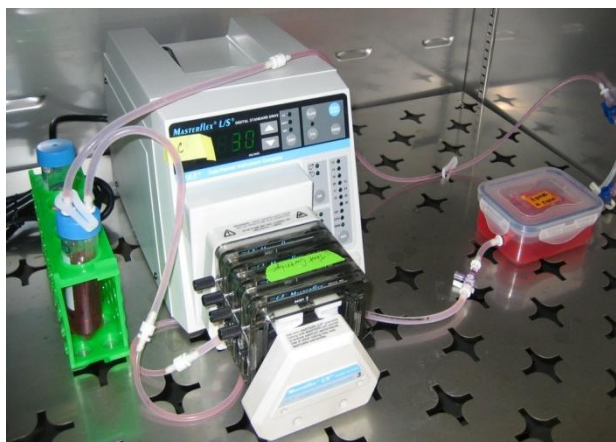


Figure 13: Overall image of the Bioreactor system set-up

Scaffold preparation

Expanded Polytetrafluoroethylene

Tubular ePTFE samples were cut to size and sutured onto barbed male fittings. After autoclave sterilization, the ePTFE scaffolds were placed in 70% ethanol for 15 minutes, followed by 15 minutes in 100% ethanol (EtOH) for denucleation. The scaffolds were then transferred to Conditioning Media (1:6 FBS to M199 base media) and placed in a humidified incubator (37°C, 5% CO₂) for 24 to 48 hours. On the day of cell seeding, the scaffolds were installed (under sterile conditions) into the bioreactor systems and flushed via syringe lumenally and then transmurally with Conditioning Media. At this point, the bioreactors were attached to an 8-roller peristaltic pump, and Conditioning Media was pumped transmurally through the scaffolds for 20 minutes at 150 r.p.m. to complete the conditioning process.

Poly Lactic-co-Glycolic Acid

PLGA scaffolds were obtained by the electrospinning team of the Cal Poly Tissue Engineering lab as a 4mm or 3mm diameter 12cm long mandrel. Scaffolds were cut to the appropriate length, 3cm-4cm as determined by the fittings inserted into bioreactors. Cut scaffolds were then sutured onto luer lock fittings with silk suture.

Mounted scaffolds were then placed in 8 ml of 70% EtOH for 30 minutes. Scaffolds were removed with sterile forceps and placed in a 50ml trough reservoir of sterile DCF-PBS. Using a 10ml syringe, 3ml of DCF-PBS was then flushed through the scaffold lumenally followed by an additional 3ml transmurally. Scaffolds were then moved to a sterile trough reservoir of Conditioning media. Again, using a 10ml syringe, 3ml of Conditioning Media was flushed through the scaffold lumenally followed by transmurally. Scaffolds were then mounted into

bioreactors filled with Bioreactor Media, and a lid was placed on top. Throughout this process, scaffolds were kept sterile by using sterile equipment and the appropriate sterile technique.

Bioreactors were then connected to 50ml reservoirs, filled with conditioning media, via tubing, sealing the entire system from exposure to contamination. The systems were taken into a humidified incubator and loaded onto an 8 roller masterflex pump. The distal two-way valve was then shut to encourage transmural flow of the media through the scaffold. The pump was turned onto 150 r.p.m. and left for 6-12 hours to condition the PLGA scaffolds with the proteins found in conditioning media.

Cell Sodding and Flow Ramp-Up

Cells (both HUVEC's and HUVSMC's) were trypsinized at approximately 80% confluence and centrifuged into a pellet. The extra solution was aspirated off and the pellet was resuspended in cell specific culture media at a concentration of approximately 3.3 million cells per milliliter.

This cell solution was then pressure sodded onto the scaffold by syringe injection into the system transmurally, followed by a chase with 3mL of cell specific culture media to ensure that all cells had cleared the components between the injection port and the scaffold. For both polymers, the desired sodding density is 1.5×10^6 cells/cm²², where the surface area of interest is the luminal surface of the scaffold.

In ePTFE single sod set-ups, only HUVECs were sodded onto the graft, followed by one hour of transmural flow at 10 r.p.m., one hour of luminal flow at 10 r.p.m., and approximately 12 hours of luminal flow at 15 r.p.m. At that point, flow was increased by 10-15 r.p.m. every hour until a flow of 90 r.p.m. was achieved. See Appendix A for complete protocol.

For PLGA single sod set-ups, again only HUVECs were sodded onto the scaffold, however unlike ePTFE, this step was followed by only 10 minutes of transmural flow at a pump setting of 10 r.p.m. This was followed by a rest period of 3-6 hours, after which luminal flow was gradually increased by 10-15 r.p.m. every 2-3 hours until a flow of 90 r.p.m. was achieved. See Appendix A for a complete PLGA single sod protocol.

In dual sod set-ups for both polymers, HUVSMC's were sodded first, followed by a 10 minute, 10 r.p.m. transmural flow period and a 1 to 3 hour rest period. After the rest period, HUVEC's were seeded in a similar fashion. At the end of the second 1 to 3 hour rest period, luminal flow at 15 r.p.m. was initiated for 3 to 6 hours, after which flow was increased by 10-15 r.p.m. per hour until a flow of 90 r.p.m. was achieved. See Appendix A for complete PLGA dual sod protocol.

After sodding, bioreactors and pump were placed in a large incubator at 37°C and 5% CO₂, where they remained for the duration of the set-up. All procedures were carried out to ensure sterility of the bioreactor and media reservoir system.

Take Down and Evaluation

At designated time points, bioreactors were removed from the incubator and scaffolds were cut out of the system, and were either placed in 15mL conicals and fixed in liquid nitrogen by slowly submerging the conical for gene expression analysis, or placed in 1 of three fixatives overnight, histochoice, 10% formalin, or 2.5% glutaraldehyde. For later experiments, PBS was perfused through the scaffold prior to removal from the bioreactor in order to eliminate any residual cell culture media that would interfere with downstream gene expression analysis. After fixation, scaffolds were placed in a -80°C freezer until evaluation. En face bisbenzimidazole (BBI, diluted 1:1000 in Milli-Q water) staining and scanning electron microscopy (SEM) were initially carried out to evaluate cell presence and cell coverage on select scaffolds in order to validate the BVM

protocol. After poor initial results, the protocol was changed such that the proximal end (~1cm) of each BVM was removed and fixed in gluteraldehyde for BBI and SEM imaging (Appendix B). For dual-sodded PLGA vessels, a previously established cell tracker protocol was implemented into the cell culture stage prior to sodding, to allow for the distinction of both cell types during fluorescent imaging, this protocol can be found in Appendix B.

Gene Expression Methods

RNA Isolation

Scaffolds were taken out of bioreactors and cut from their mounting barbs. They were then flushed with 5mL PBS transmurally to remove any residual media. Scaffolds were then each placed in a 15mL conical and submerged in liquid nitrogen for 5-10 minutes. The conicals were then stored at -80°C until RNA isolation was performed.

Without allowing the scaffold to thaw, scaffolds were cut to roughly 1 cm pieces to fit into a 2 mL microcentrifuge tube. An RNeasy Mini Kit (Qiagen, Catalog No 74104) was used to isolate RNA from BVMs as follows. 600 uL of Buffer RLT, a cell lysate, was pipetted into the microcentrifuge tube with the scaffolds. Tubes were then closed and agitated by shaking and vortexing for 5 minutes. Lysate was then removed and pipetted directly into a QIAshredder spin column in a 2mL collection tube, and then centrifuged at 161000 rcf for 2 minutes.

600uL of 70% ethanol was then mixed with the lysate flow through and half of the ethanol and lysate mixture was pipetted into a RNeasy spin column in a 2 mL collection tube. The mixture was then centrifuged for 15 seconds at 161000 rcf Flow through was then discarded and the remainder of the ethanol lysate mixture was added to the RNeasy spin column, the centrifugation process was repeated and flow through was discarded. 700uL of Buffer RW1 was then added to the spin column and centrifuged for 15 seconds at 161000 rcf, flow through was then discarded.

500uL of Buffer RPE was then added to the RNeasy spin column and centrifuged for 15 seconds at 161000 rcf. Flow through was discarded and an additional 500uL of Buffer RPE was added and centrifuged for 2 minutes at 161000 rcf. The spin column and microcentrifuge tube were then spun again at 161000 rcf for 1 minute. The RNeasy spin column was then placed in a new 1.5 mL collection tube and 30uL of water was pipetted directly onto the spin column membrane. This was then centrifuged for 1 min at 161000 rcf. The flow through was then pipetted back onto the membrane to ensure the collection of all the RNA, and spun again for 1 min at 161000 rcf. A bulleted protocol for RNA isolation is found in Appendix C.

To quickly assess the concentration and quality of the mRNA isolated from the scaffolds, a NanoDrop 2000 spectrophotometer was employed. The NanoDrop software outputs the concentration and the ratio of 260nm/280nm and 260nm/230nm light absorbance, which are used to get an idea of RNA quality and purity where protein contamination will decrease the 260/280 ratio and other organic contaminants can alter the 260/230 ratio. Ideally, both the 260/280 and 260/230 ratios would be 2.0, though successful qPCR was carried out on samples with ratios as low as 1.73 and 1.41 respectively. Finally, the concentration must be greater than 200ng/μL for use in downstream qPCR analysis. The full protocol for using the NanoDrop spectrophotometer and system can be found in Appendix C.

Reverse Transcription

Complimentary DNA (cDNA) was Reverse Transcribed from the RNA samples using one of two kits:

Using an Accuscript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies, Catalog No #200820) cDNA synthesis was as follows:

First, the volume of the sample needed to reach the 2000ng target amount of template in each cDNA reaction was determined. This was done by dividing 2000ng by the concentration of RNA in each sample. Next the amount of reactions that could be made with a given sample was determined by dividing the total volume of sample (typically 25uL) by the volume of sample needed from each tube.

Next, amount of Accuscript RT Buffer, gene specific primer, dNTP and RNase-free water needed for a master mix were determined for each RNA sample or template. For a single reaction the amounts needed are as follows: 2uL Accuscript RT Buffer, 1 uL gene specific primer, .8uL dNTP, and water calculated to be 12.8uL minus the volume of sample needed. The single reaction amounts along with the single reaction amounts for template were then multiplied by the number of tubes to be made for each sample and with the addition of 10% of each for pipetting errors. The master mix was then prepared by first adding water, the buffer, primer and dNTP, followed by the template.

Once the master mix was prepared, 16.5uL of the mix was pipetted into each PCR tube. The tubes were then placed in the thermal cycler for 65°C for 5 minutes and 20°C for 3 minutes. Next a second master mix was prepared by adding 2uL DTT, 1uL AccuScript RT, and .5uL of RNase Block ribonuclease inhibitor for each tube needed, plus one extra for error. When the thermal cycler finished, 3.5uL of the master mix was added to each tube, and each tube was centrifuged briefly to ensure all contents were at the bottom.

The samples were again loaded into the thermal cycler and run at 42°C for 60 minutes, then 72°C for 15 minutes, then 4°C until they were taken out and stored at -20°C.

Using an AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, Catalog No200436) cDNA synthesis was as follows:

Again, the amount of template needed for each reaction to end with 2000ng of template and total number of tubes to be made were calculated as described in the above section.

Next the first master mix was made by multiplying the following recipe for 1 reaction by the number of tubes needed and adding an additional 10 percent for pipetting errors. A single reaction requires 3uL of random primers, the calculated amount of template and 12.7uL minus the volume of template needed for RNase free water. The mix was then made by adding water first, followed by primer and template. After the master mix was made, 15.7uL of the mix was added to each PCR tube. The tubes were then placed in the thermal cycler and incubated at 65°C for 5 minutes, then allowed to cool to room temperature for 10 minutes.

A second master mix was then prepared by multiplying the single reaction recipe by the number of tubes to be made plus one. A single reaction consists of 2uL of AffinityScript RT Buffer, .8uL dNTP, .5uL RNase Block Ribnucleae Inhibitor, and 1uL of AffinityScript Multiple Temperature RT. All the components were mixed together in a tube and then 4.3uLof the mix was alloquated into each PCR tube.

Since random primers were used, the reaction was then incubated at 25°C for 10 minutes in the thermal cycler to extend the primers. PCR tubes were removed from the thermal cycler and reaction components were gently mixed by pipetting up and down. PCR tubes were then placed back in the thermal cycler and reactions were incubated at 42°C for 60 minutes followed by 70°C for 15 minutes. Upon completion of cDNA synthesis, tubes were stored at -20°C until need for qPCR. A bulleted protocol for Reverse Transcription can be found in Appendix C.

Quantitative PCR

Primer Design

In order to carry out gene expression analysis, a set of primers for specific genes was developed.

The genes of interest for this application are summarized in Table 1 below.

Table 1: Gene of Interest Table

Gene	Function
PECAM	Endothelial Cell Marker
ICAM	Inflammation Marker
Ki-67	Proliferation Marker
GAPDH	Reference
Bax	Pro-Apoptosis Marker
XIAP	Anti-Apoptosis Marker

The key considerations involved in primer design for qPCR include primer length, product (amplicon) length, annealing temperature, and GC base pair content. For qPCR, the goal is to use short, highly specific primers with product sizes from 180 to 220 base pairs and annealing temperatures all within 2°C of all other primers (normally 60°C \pm 1°C). Because GC base pairs play a key role in annealing temperature, primer selection based on GC content is common, where this primer set uses a range of 30% to 80%.

The first step in primer design is gene and mRNA sequence identification. The NIH-funded GenBank website contains mRNA sequences for thousands of genes spanning multiple species. For this application, human mRNA sequences for the genes mentioned previously were obtained, using the last half of the sequence, or last third for longer sequences, for primer selection as this region of the mRNA is generally the most specific.

These sequences were then entered into the Primer3 primer design program. Settings were changed according to the protocol found in Appendix C, and primer sequences were identified.

These primers were then run through the Primer-BLAST program (also an NIH tool) to check for homology with different transcript variants of the same gene as well as homology with non-target genes. If the specificity of the primers was too low (defined as less than 5 mismatches with non-target genes), or excluded too many transcript variants, new primers were picked from Primer3 and the same process was carried out until suitable primers were identified.

qPCR Set Up

During all steps of qPCR, precautions were taken to keep the qPCR plate and components of the reaction cold throughout preparation. First, a plate set-up determining where each primer and sample were to be placed in a 96 well plate was created. For each sample, three replicates of each primer were made for each of six primers, totaling 18 reactions per sample. A master mix was made for each primer, for each sample. Each master mix contained 41.25ul SybrMM (Applied Biosystems Catalog No 4309155), 2.475uL primer, 13.2uL cDNA template, and 25.75uL of RNase free water. Master mixes were made in a horizontal laminar flow station by first adding water, primer, template, and lastly Sybr. Once made, 25uL of master mix was then allocated into the predetermined wells of the 96 well plate. Next, non-template controls were made, one for each primer, by adding 11.75uL of RNase free water, .75uL of primer, and 12.5uL of SybrMM to the designated wells on the plate.

The plate was then taken to the Applied Biosystems 7500 Fast Real-Time PCR System and the 7500 Fast System SDS Software on the laptop was set to run in two stages. Stage 1 consisted of 1 repetition of 95°C for 15 minutes and Stage 2 consisted of 40 repetitions of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Lastly a dissociation step was added and the sample size was changed from 20uL to 25uL.

The qPCR cycle was then run and fluorescence versus cycle number amplification plots was generated for each well by the program. These plots were then analyzed and the Cycle threshold(Ct) was set as the point of steepest slope, indicating the fastest amplification time. These Ct values were then exported to excel and the 3 replicates of each primer were averaged to obtain Ct values for each primer for each sample.

Gene expression analysis requires a ΔCt value in order to compare different treatments, where ΔCt is obtained by subtracting the average ΔCt of the reference gene (in this case GAPDH) from the average ΔCt of the gene of interest³⁸. Equation 1 below illustrates this process.

$$\Delta Ct = Ct_{avg \text{ sample}} - Ct_{avg \text{ reference}} \quad \text{Equation 1.}$$

In order to compare one treatment group to another, in this case stented versus unstented BVMs, the differences in ΔCt values, or $\Delta\Delta Ct$, must be calculated. This process involves subtracting the ΔCt value of the treatment from that of the control, and then plugging the obtained $\Delta\Delta Ct$ value into Equation 2 to obtain a fold change.

$$\text{Fold difference in treatment} = \frac{\Delta Ct_{\text{control}}}{\Delta Ct_{\text{treatment}}} \quad \text{Equation 2.}$$

In the case of the BVM, the control will be an unstented BVM from the same time point as the stented BVM. Once the baseline (or unstented BVM) gene expression at a given time point has been established, there is no need to repeat the analysis and the results may be employed in all subsequent experiments involving stents. A qPCR bulleted protocol can be found in Appendix C.

CHAPTER 3: Results

PLGA BVM Development Results

Set up #1

A total of 4 vessels were created using 3.5cm length x 4mm diameter PLGA scaffolds, two 3 day time points and two 6 day time points, and only HUVECs were used. The goal of this experiment was to determine the efficacy of a sterilization process, 30 minute scaffold soak in 70% Ethanol, and also to experiment with pump settings. Flow began a 20 minutes after the initial sodding step, and gradually increased by 10-15 r.p.m. 3 hours at a time to reach 90 r.p.m. BBI nuclear staining showed patchy cell deposition at 3 days and nearly no cells at 6 days (Figures 14 and 15). When cell coverage was present, it was of an irregular morphology as shown through SEM imaging (Figures 16 and 17).

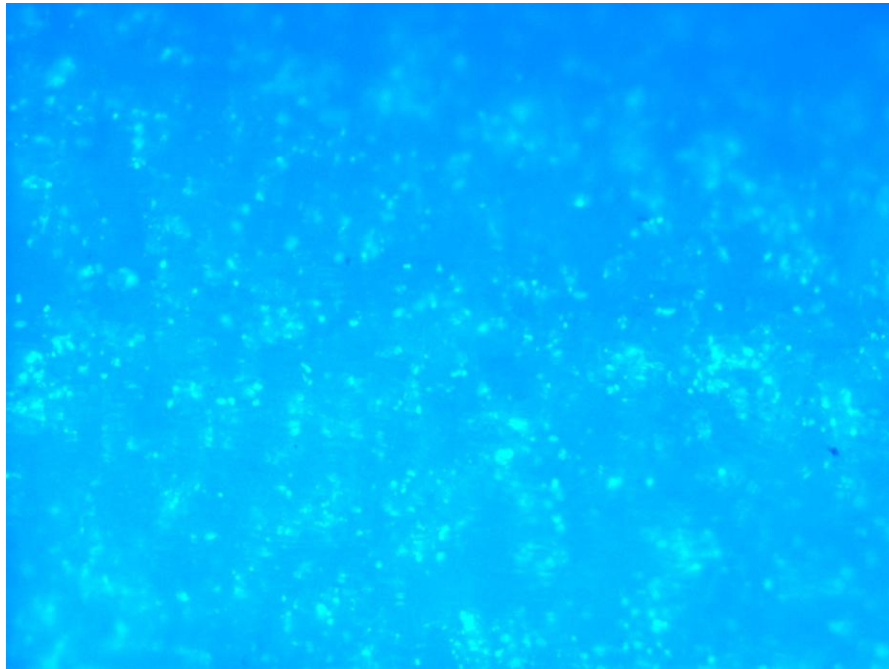


Figure 14: 10x BBI image of PLGA & HUVEC BVM at 3 days showing patchy cell coverage

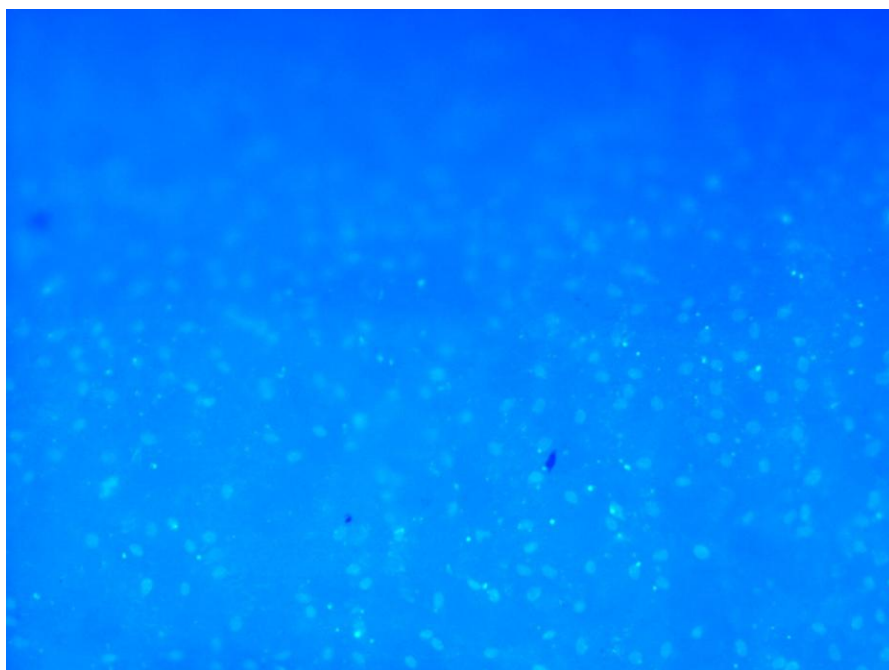


Figure 15: 10x BBI image of PLGA & HUVEC BVM at 6 days showing minimal cell coverage



Figure 16: SEM image of 3 day PLGA & HUVEC BVM at 1800x

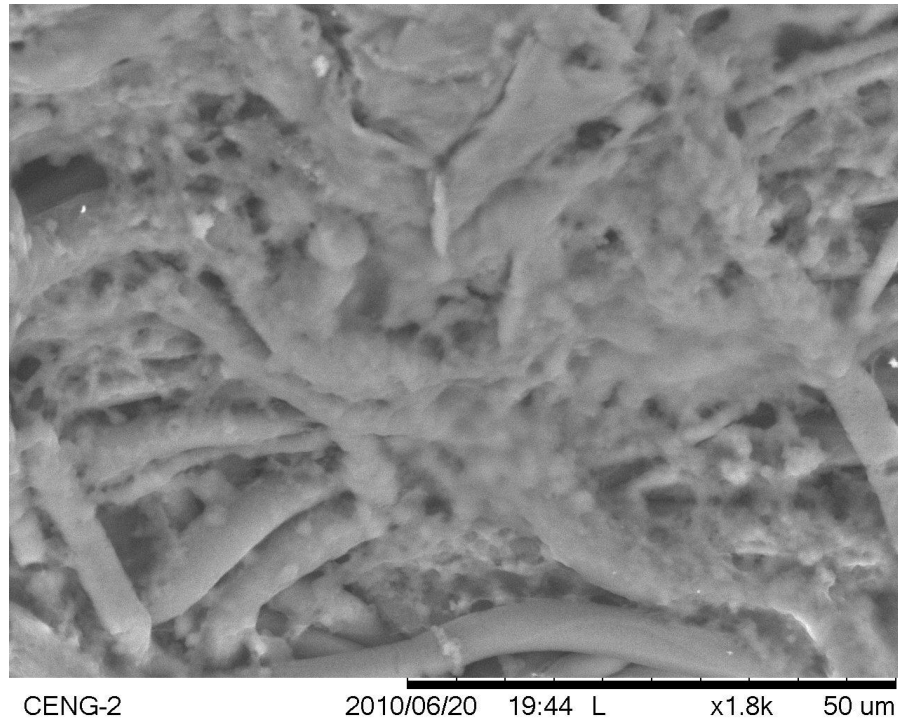


Figure 17: SEM image of 6 day PLGA & HUVEC BVM at 1800x

Set Up #2

The second set up used three 3cm x 4mm PLGA scaffolds with HUVECs only to examine a varying flow regime with time points of 3 days, 7 days, and 10 days. After seeding, cells were left to adhere for 6 hours prior to the introduction of flow. However, even with increased time to adhere, cell confluency was much lower than desired, as seen from BBI images (Figures 18-20) and SEM images (Figures 21-23).

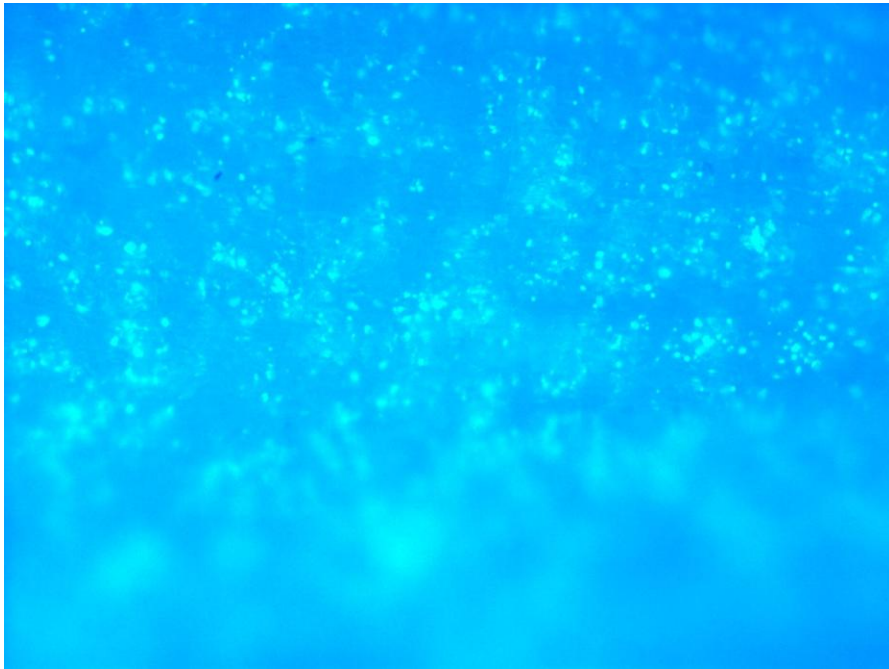


Figure 18: 10x BBI image of 3 day PLGA & HUVEC BVM



Figure 19: 10x BBI image of 7day PLGA & HUVEC BVM

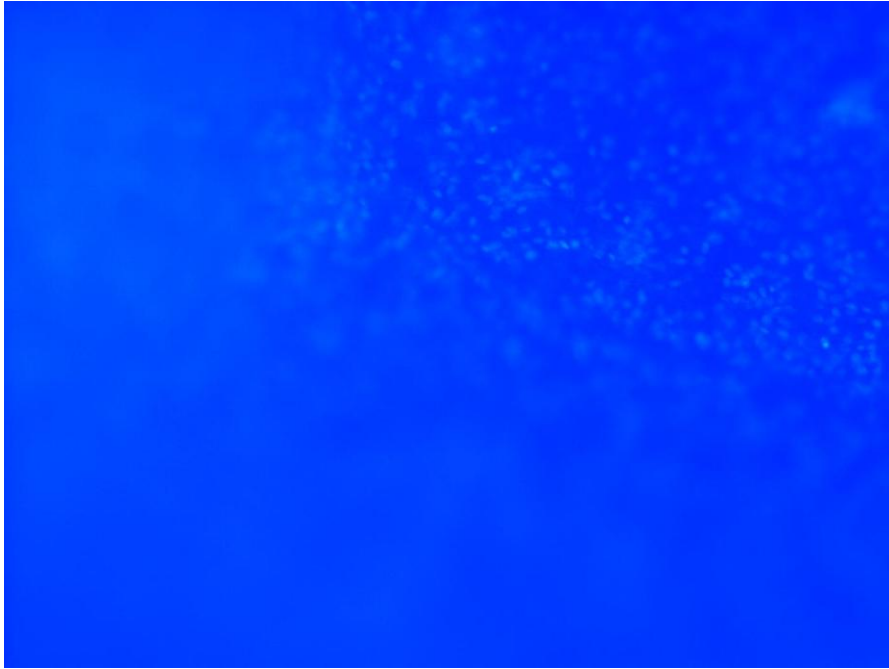


Figure 20: 10x BBI image of 10day PLGA & HUVEC BVM

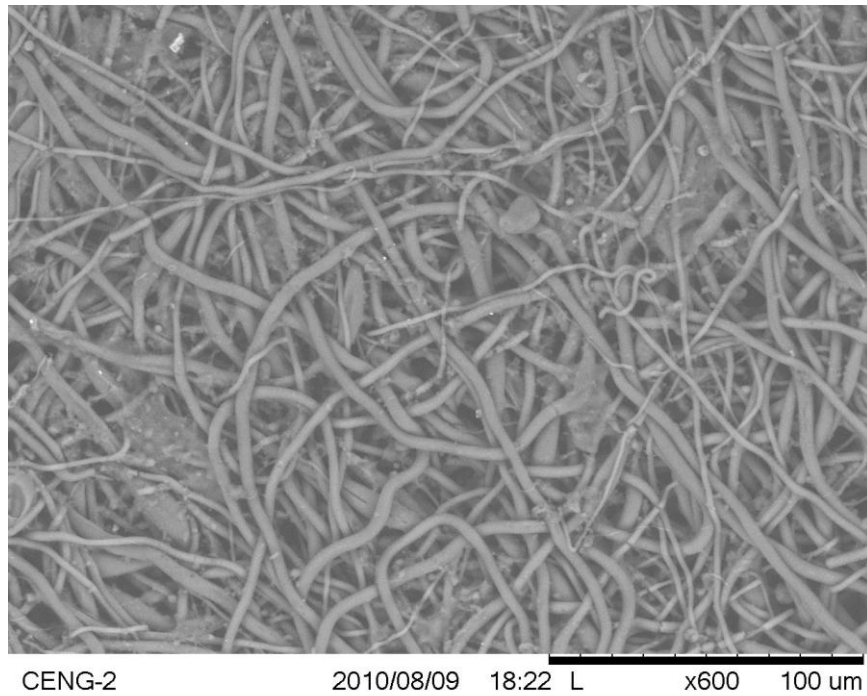


Figure 21: SEM image of 3 day PLGA & HUVEC BVM at 600x

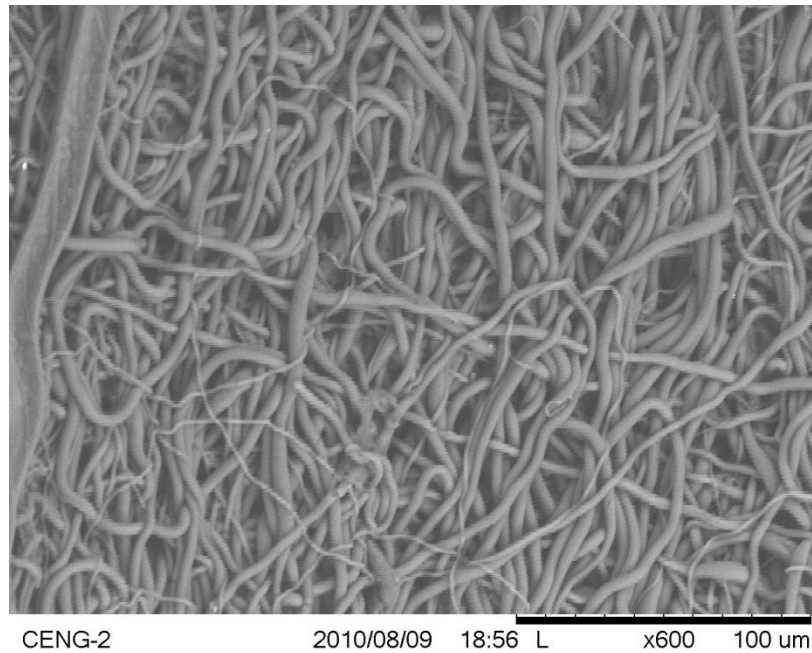


Figure 22: SEM image of 7day PLGA & HUVEC BVM at 600x

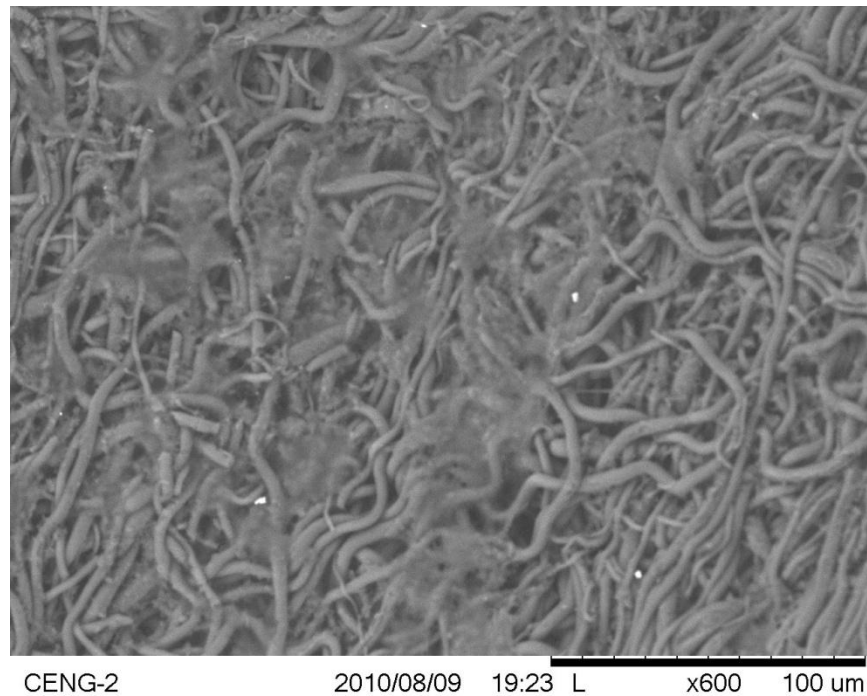


Figure 23: SEM image of 10 day PLGA & HUVEC BVM at 600x

Set Up #3

After poor results were seen with the previous set ups, it was determined that sodding vessels with HUVSMCs prior to the HUVEC sodding may increase the adhesion of HUVECs to the BVM. For this set up four 3cm x 4 mm PLGA scaffolds were used, and HUVSMCs were sodded 6 hours prior to endothelial sodding. Set ups were originally designed to last 6 and 10 days, however, because of leaking reservoirs, two vessels were taken down after 24 hours, and due to contamination of the remaining 10 day vessel, only a 6 day BVM lasted for the designated time point. SEM images from 1 and 6 days showed vastly improved cell coverage from single sodded BVMs (Figures 24 and 25). Due to microscope issues, no fluorescent images were able to be taken.

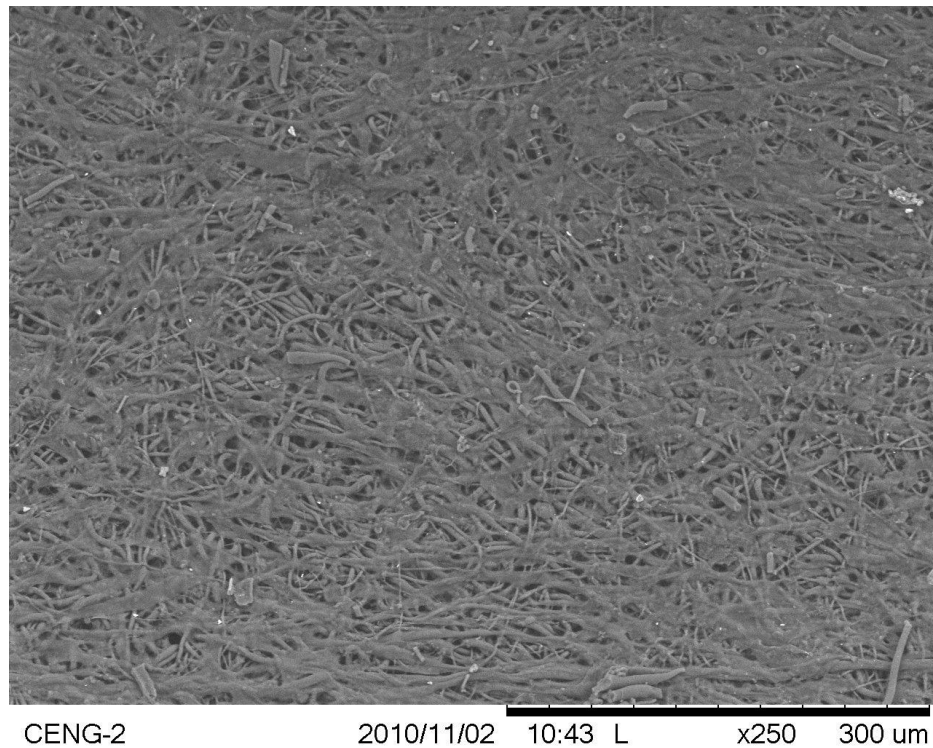


Figure 24: SEM image of dual-sodded PLGA at 1 day at 250x

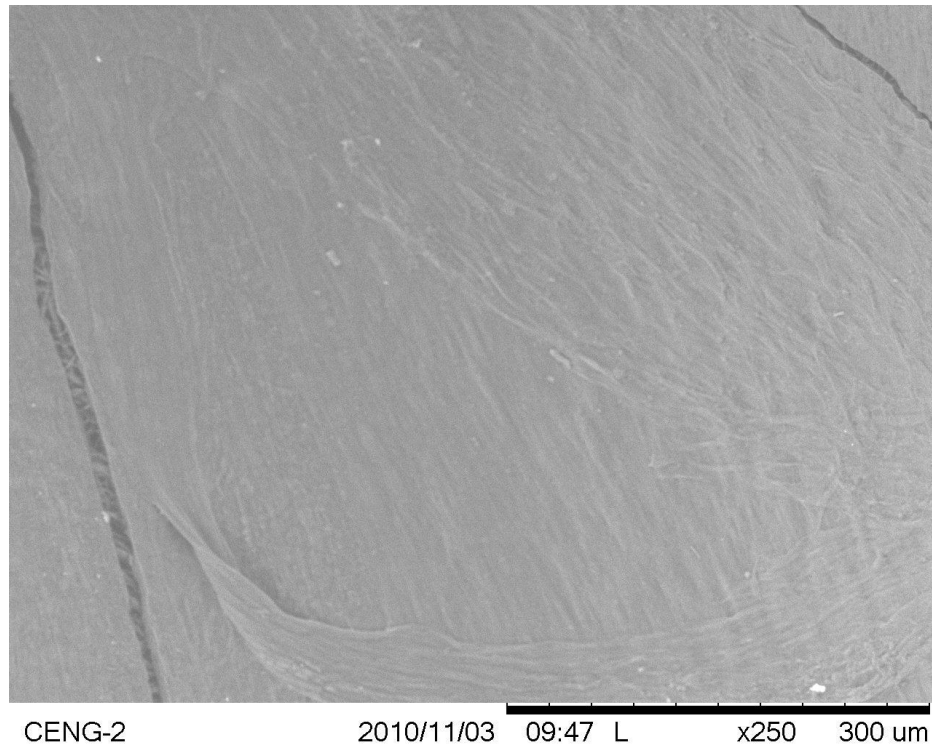


Figure 25: SEM image of dual-sodded PLGA at 6 days at 250x

Set Up # 4

The goal of set up 4 was to obtain more samples at the longer time point than the previous set up. Three 3cm x 4mm PLGA scaffolds were used, one 6 day time point and two 12 day time points. Massive contamination was seen in all vessels at six days. Growths clogged tubing, made the cell media thick and discolored, and in the bioreactor chambers themselves, fungal growth was seen. Cell tracker images showing were hard to obtain due to the large amount of contamination, but indicated cell coverage (Figures 26 & 27). SEM images were not obtained due to the contamination that was resting inside of all the BVMs.

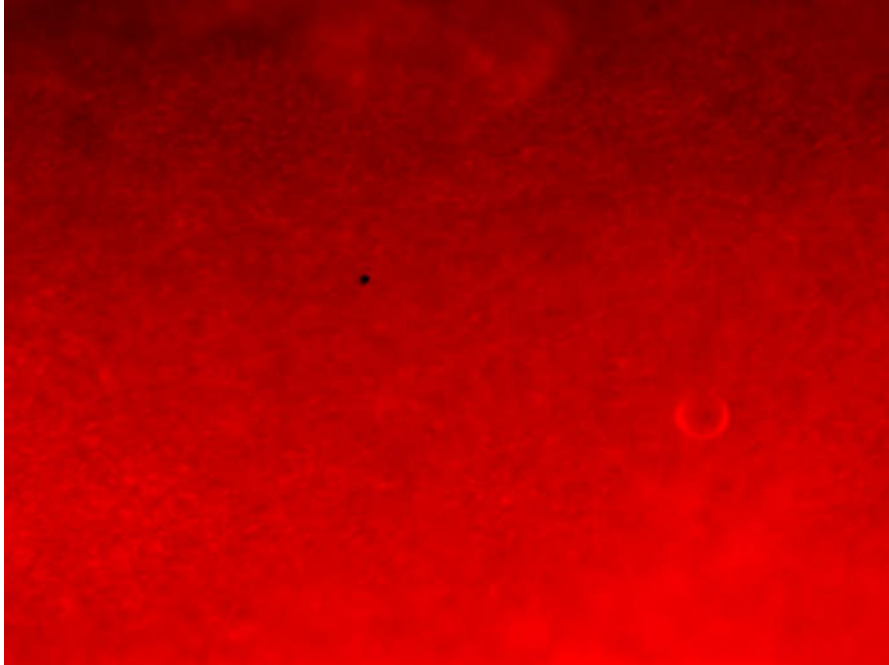


Figure 26: 10x Cell Tracker Red image of HUVECs of a dual-sodded 6 day BVM

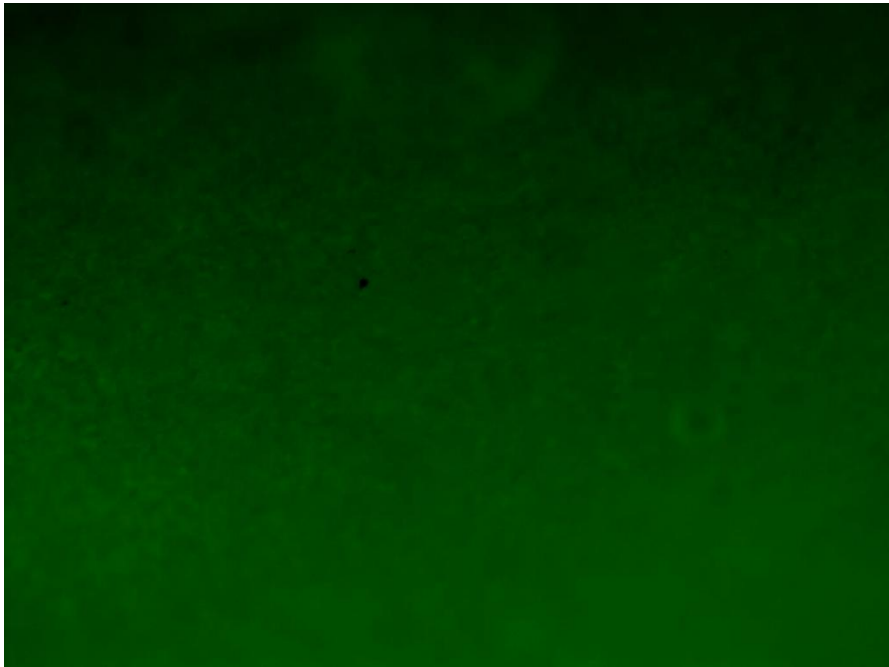


Figure 27: 10x Cell Tracker Green image of HUVECs of a dual-sodded 6 day BVM

Set Up #5

The aim of set up 5 was to replicate the positive results seen by the initial dual sod experimentation but eliminate the contamination issues. It was determined that contamination might be largely due to the prolonged exposure of the sodding port, and a stop cap was placed over the port in hopes to prevent the contamination. For this set up three 3cm x 4mm scaffolds were used, and again sodded with HUVSMCs 3 hours prior to HUVEC sodding. Time points were 2, 7 and 9 days, no contamination was seen out to 9 days, due to the addition of the stop cap. However, at the 6 day mark, CO₂ ran out and media turned basic for an unknown amount of time before it could be replaced. Due to this, good confluency results are seen at the 2 day time point, but seem to decline after. Cell tracker images show patchy results starting at the 7 day time point (Figure 28-30), and SEM images follow a similar trend (Figures 31-33).

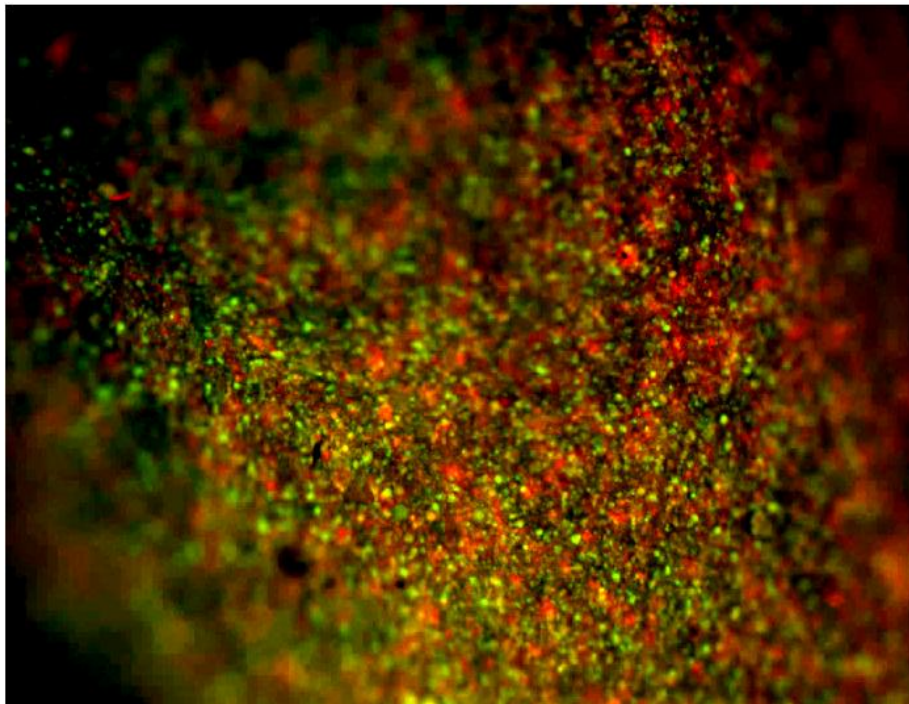


Figure 28: Image J merged image of Cell Tracker Red (HUVEC) and Cell Tracker Green (HUVSMC) of a 2 day PLGA BVM

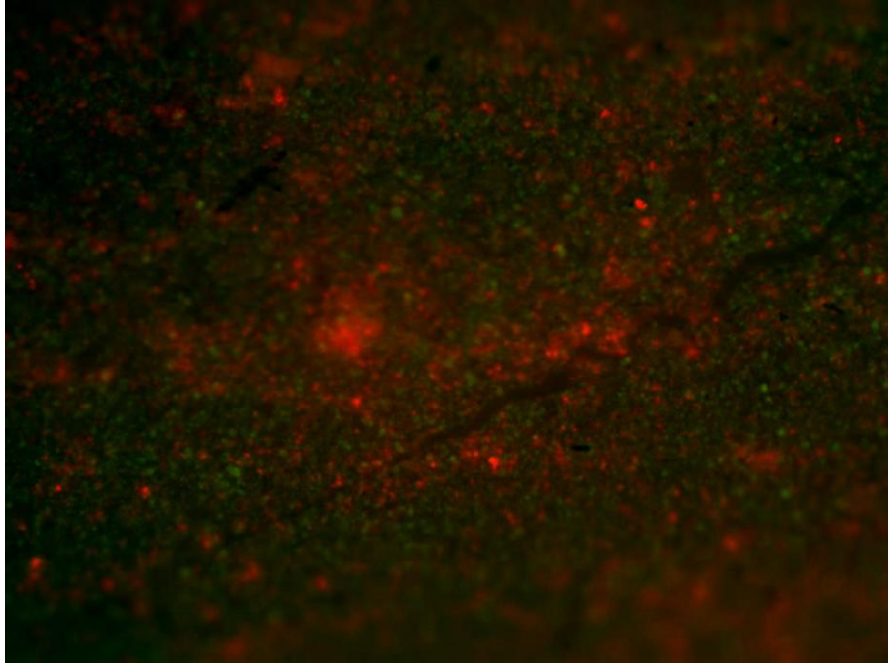


Figure 29: Image J merged image of Cell Tracker Red (HUVEC) and Cell Tracker Green (HUVSMC) of a 7 day PLGA BVM

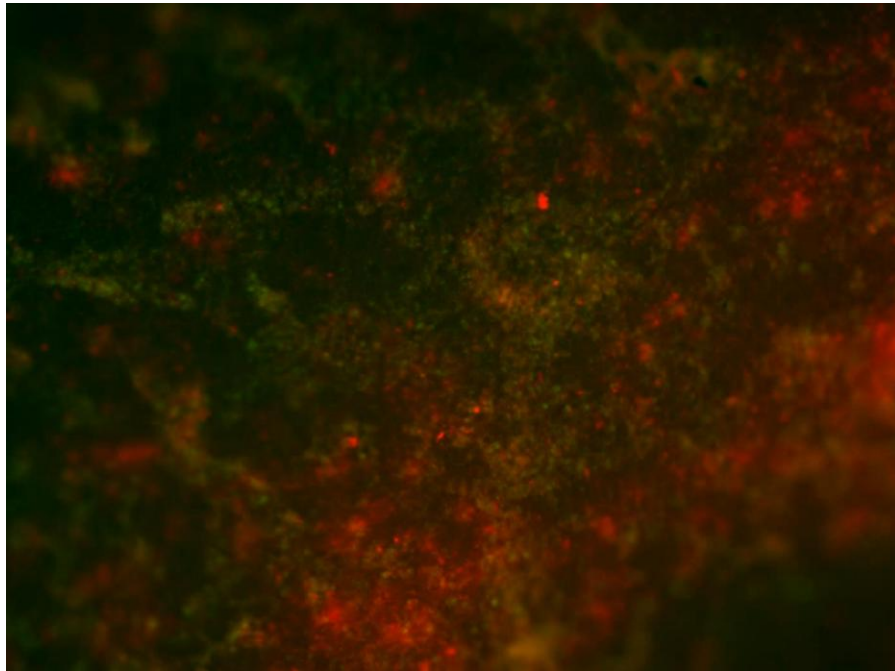


Figure 30: Image J merged image of Cell Tracker Red (HUVEC) and Cell Tracker Green (HUVSMC) of a 9 day PLGA BVM

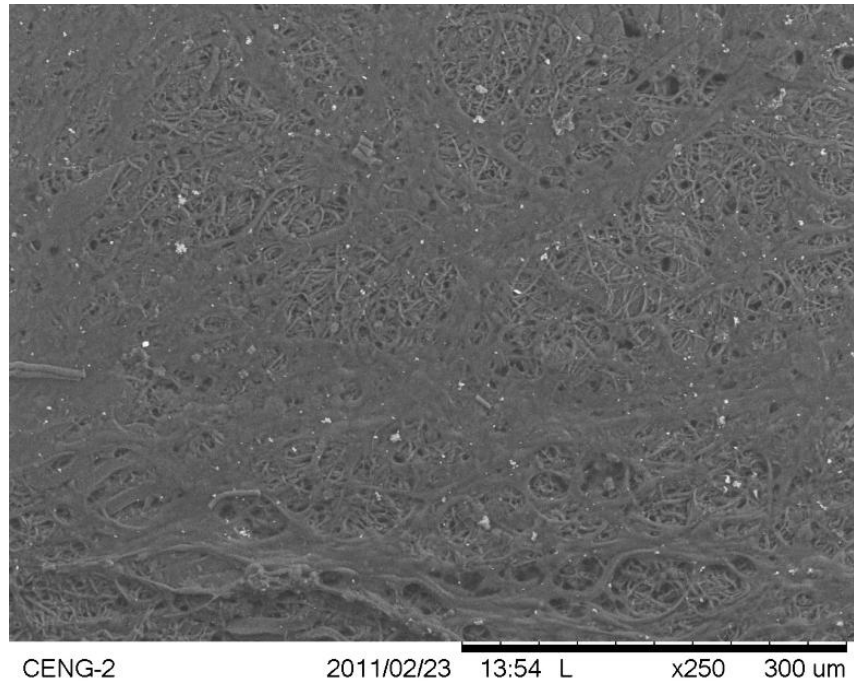


Figure 31: SEM image of 2 day PLGA Dual-Sodded BVM at 250x

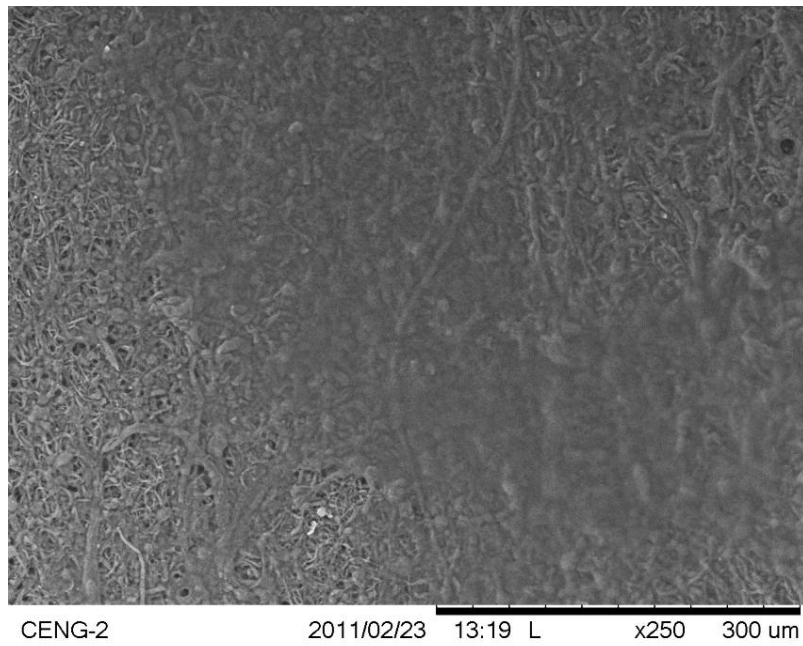


Figure 32: SEM image of 7 day PLGA Dual-Sodded BVM at 250x

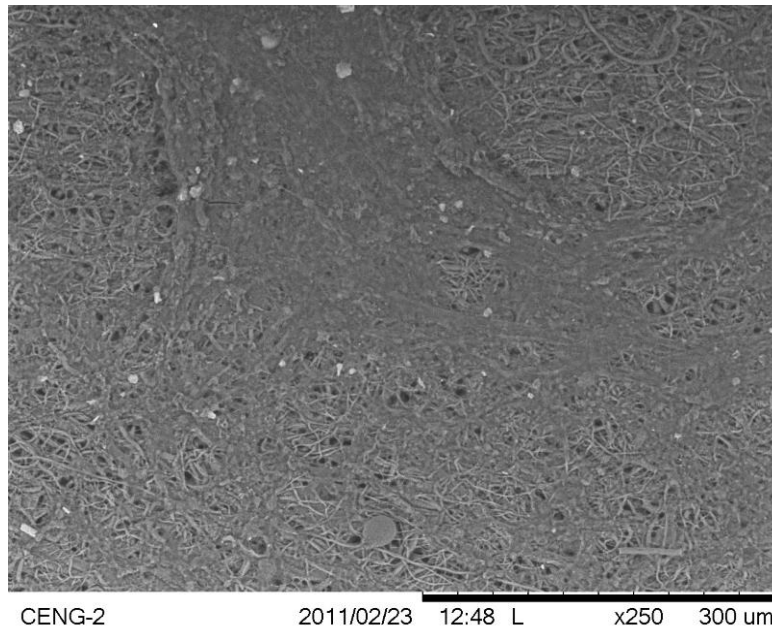


Figure 33: SEM image of 9 day PLGA Dual-Sodded BVM at 250x

Set Up #6

The purpose of the the 6th set up was to produce vessels for gene expression analysis. Four 3cm x 4mm dual-sodded PLGA vessels were made at 2 and 6 day time points. Vessels were fixed with liquid nitrogen and stored at -80°C. However, before RNA isolation could be performed at malfunction with the -80°C caused the samples to be destroyed.

Set Up #7

The purpose of the 7th set up was again to produce vessel for gene expression analysis. This time three 3cm x 4mm scaffolds were used, one dual-sodded 1 day (1A), on HUVSMC only 1 day (1B), and one dual-sodded 3 day (1C). RNA isolation was performed and only the 3 day dual sodded vessel had the quantity and quality of RNA needed. Results from this isolation can be seen in Table 3 below.

Set Up #8

The purpose of the 8th set up was again to produce more vessels for gene expression. Four 3cm x 4mm dual sodded PLGA vessels were made, with two 1 day time points (2A and 2B) and two 3

day time points (2C and 2D). RNA was isolated in the quantity and quality desired for three of the four vessels, RNA isolation data can be seen in Table 3 below.

Set Up #9

The goal for the 9th set up was to first decrease the inner diameter of the PLGA scaffold from 4mm to 3mm to create vessel that a stent could be deployed in and also to increase the time point of gene expression data to 7 days. Two 3.5cm x 3mm dual-sodded PLGA vessels were produced at a 7day time point (3A and 3C). RNA isolation was not successful for these vessels, as shown by their low concentrations in Table 3.

Set Up #10

Set up 10 had the goal of correcting what went wrong with Set Up #9. Three 3.5cm x 3mm dual-sodded PLGA BVMs were made at the 7day time point. It was necessary to repeat this to ensure that RNA isolation from the previous set up was not due to operator error. For this set up, BBI and SEM images were also taken to confirm whether or not it was the change in diameter of the scaffold that negatively affected the previous results. BBI and SEM images show a much lower confluence than previous results for dual-sodded BVMs of a 4mm diameter (Figures 34 and 35). RNA isolation again failed and results can be found in Table 3 under samples 4A, 4B, and 4C.

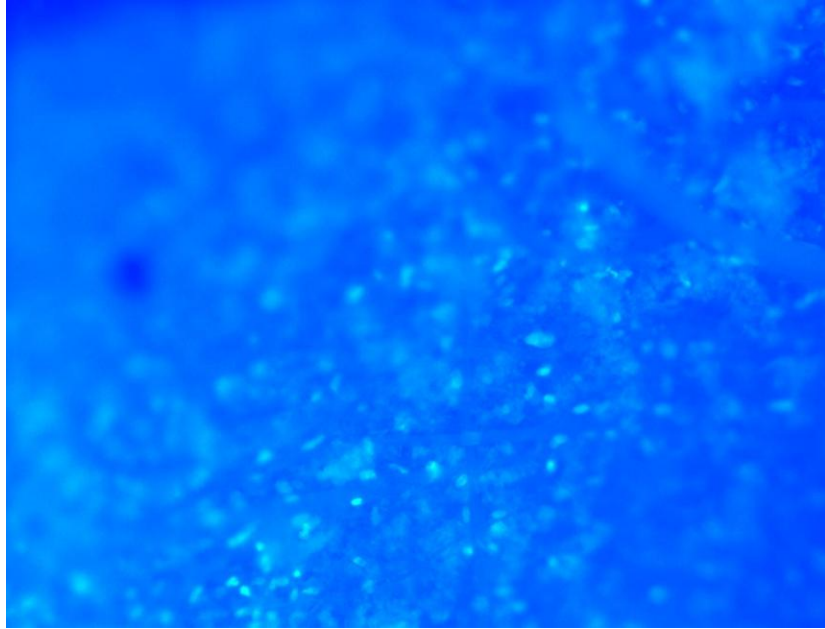


Figure 34: 10x BBI image of 3mm Dual-Sod PLGA 7 day BVM

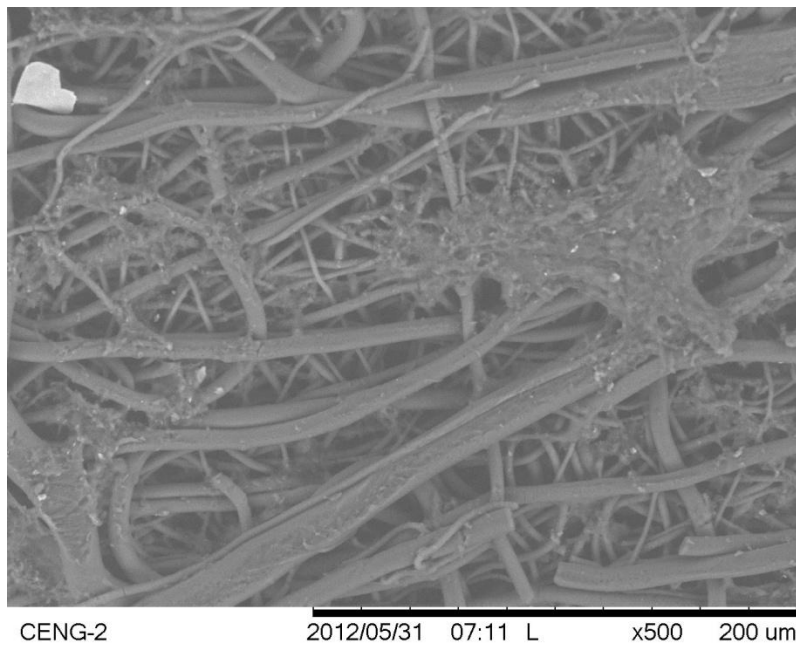


Figure 35: 500x SEM image of 3mm Dual-Sod PLGA 7 day BVM

Table 3: RNA Isolation from PLGA

Sample	Concentration (ng/uL)	260/280	260/230
1A	117.4	2.06	.82
1B	119.4	2.04	1.86
1C	400.9	2.04	2.10
2A	926.0	2.06	2.15
2B	225.9	2.06	1.61
2C	663.7	2.10	2.20
2D	181.0	2.07	.95
3A	184.6	2.05	.78
3B	23.8	2.29	.10
4A	81.9	2.04	.98
4B	89.3	2.06	1.31
4C	43.7	2.01	.71

ePTFE BVM Development Results

Set-up #1

For the first set-up on ePTFE, HUVEC's were sodded onto two identical 3.5cm x 3mm (inner diameter) scaffolds. These scaffolds were used exclusively for RNA isolation, the results of which can be found in Table 2. Unfortunately, neither scaffold yielded the concentration of RNA required for qPCR analysis, however the RNA was of good quality as assessed by gel electrophoresis (Figure 36). Concentrations and spectrophotometric ratios can be found in the summary Table 2.

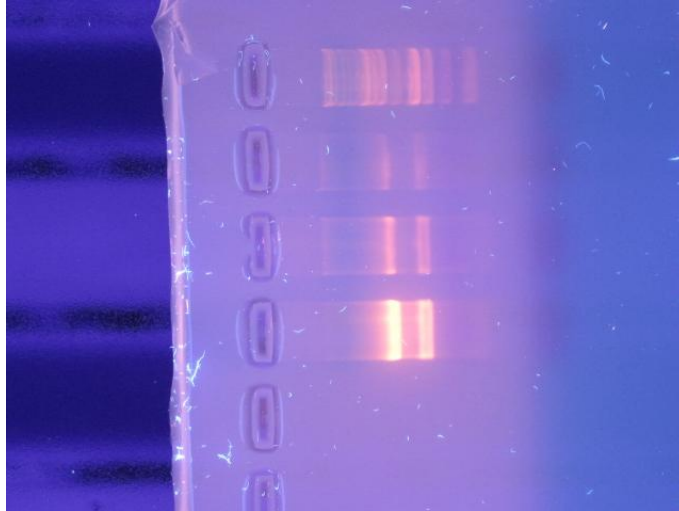


Figure 36: Gel electrophoresis (first sample under the top ladder) shows low concentration but relatively high quality RNA isolated from ePTFE scaffold

Set-up #2

The second set-up on ePTFE involved a single 3.5cm x 3.5mm scaffold seeded with HUVEC's and exposed to flow for three days. Due to the low yield from the first set-up, the purpose of this scaffold was to obtain BBI images to qualitatively assess cell deposition on the ePTFE scaffolds using the original protocol. After BBI imaging, an attempt to isolate RNA from the scaffold was carried out in order to determine whether or not RNA isolation is possible from a scaffold that has previously undergone BBI imaging. Figure 37 shows the very low cell deposition on the scaffold, which later contributed to the change of the protocol. Both the quantity and quality of RNA isolated from this scaffold were poor as assessed by spectrophotometry (results summarized in Table 2).

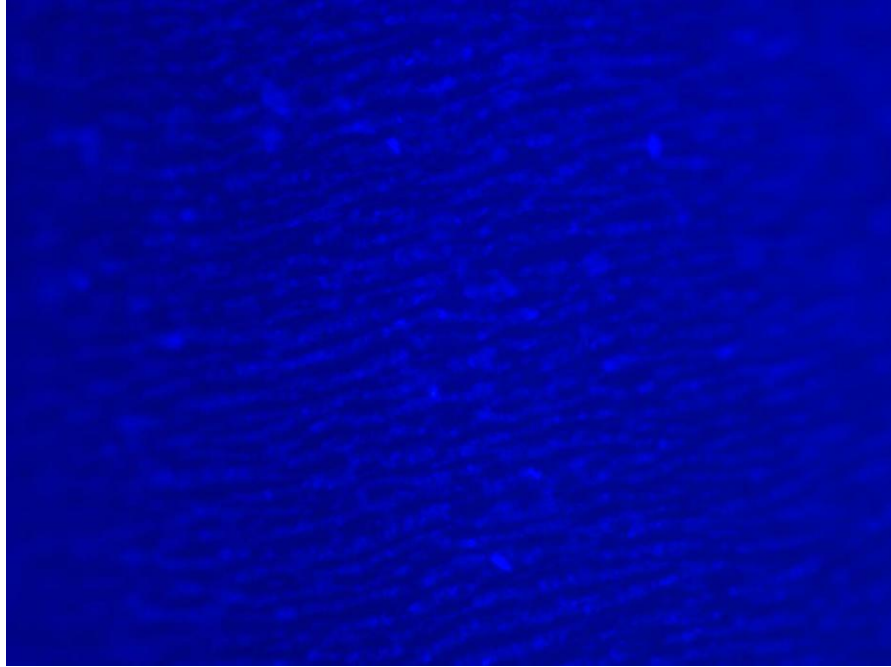


Figure 37: BBI at 10x of ePTFE sodded with HUVEC's and exposed to flow for 3 days.

Set-up #3

The third ePTFE set-up consisted of two HUVEC-sodded 5.5cm x 3.5mm scaffolds exposed to three days of flow. Due to the low RNA yields of previous isolations, the aim of this set-up was to generate scaffolds with sufficient cell numbers to overcome any issues related to cell availability. One of the two scaffolds showed a noticeable increase in RNA concentration (3A in Table 2), though still too low for qPCR analysis. The results from the other scaffold were similar to previous attempts.

Set-up #4

In an attempt to increase the efficiency of cell sodding on the ePTFE, the dual sod procedure developed for PLGA was implemented using HUVECs and HUVSMCs on a 3.5cm x 3.5mm scaffold. This 24 hour set-up was primarily an opportunity to evaluate the dual sod procedure on ePTFE and to gain experience working with two different cell types. The BBI images of the scaffold, seen in Figures 38 and 39, show a noticeable increase in cell deposition compared to the

earlier, single sod vessel from the second set-up. It is important to note that this set-up involved approximately twice the number of cells as the second set-up, and that the HUVSMCs should adhere better and help the HUVECs adhere better than the HUVECs do alone. The SEM images confirmed what the BBI images suggested, which is that we were unsuccessful in developing a monolayer of cells on the scaffold. In Figure 40, we see only isolated patches of cell coverage with the majority of the scaffold devoid of any kind of cell layer. Figure 41 shows individual cells covering the ridges of the ePTFE scaffold.

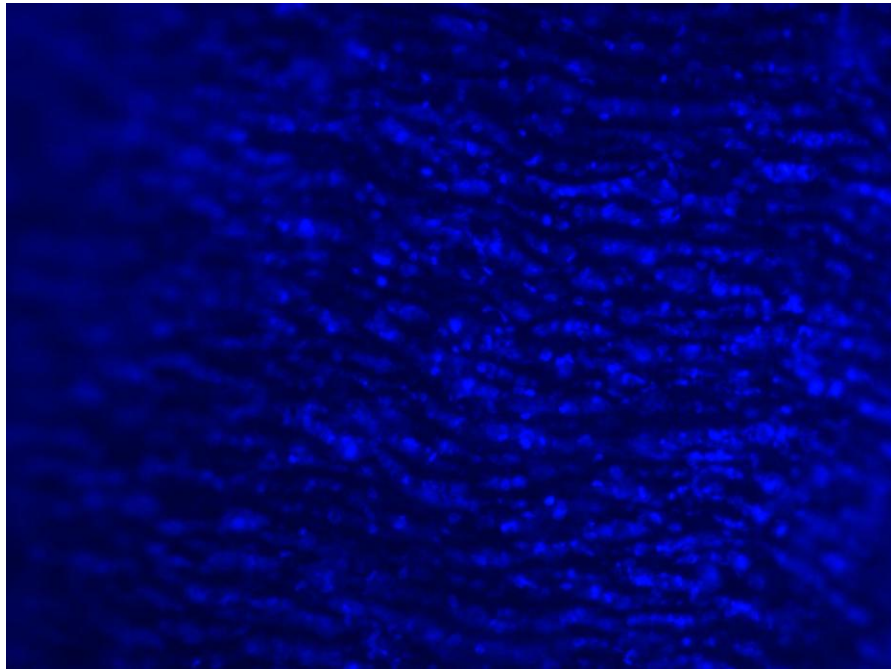


Figure 38: BBI image of 24 hour HUVEC + HUVSMC on ePTFE

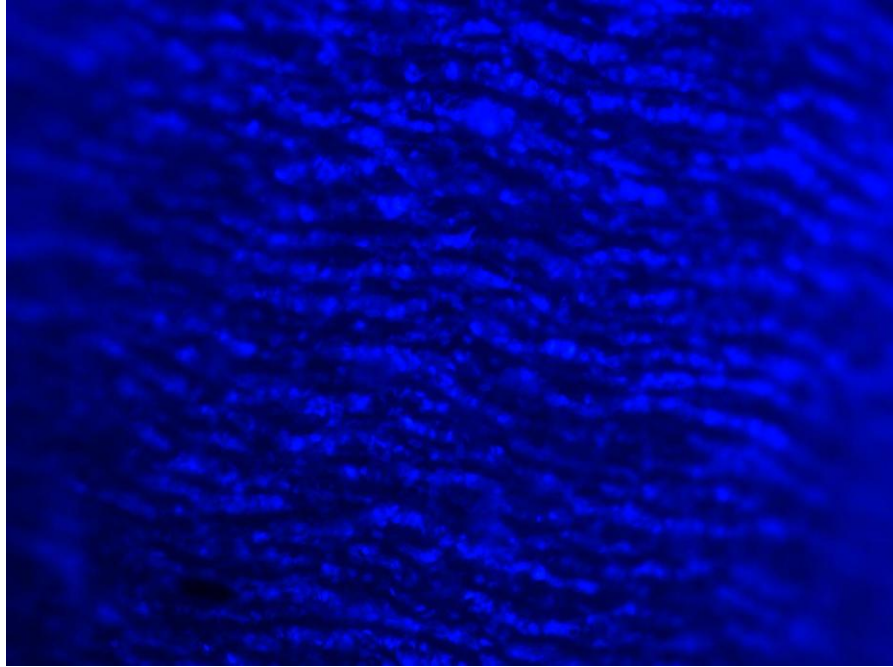
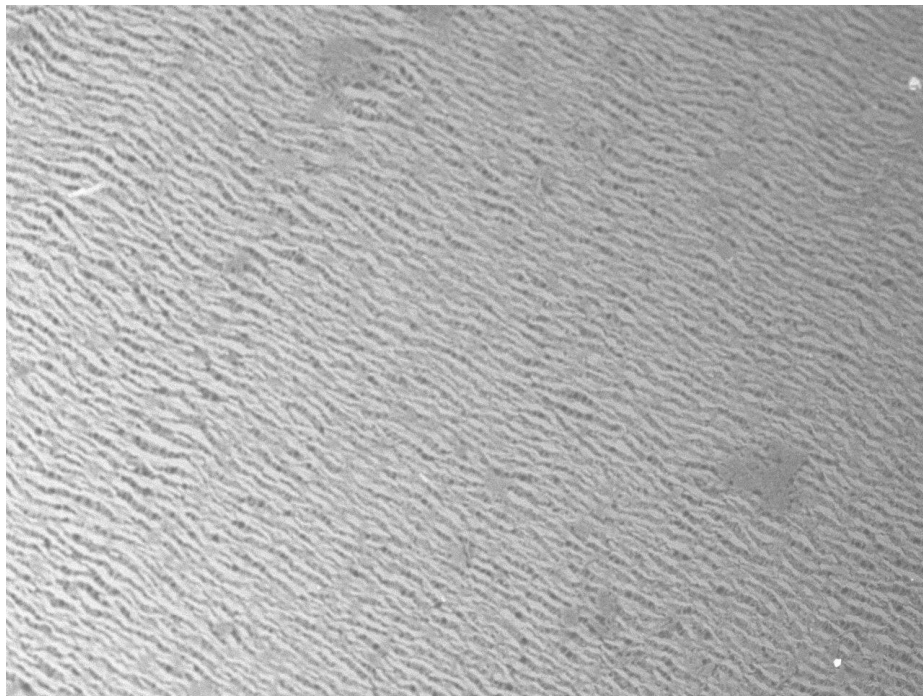


Figure 39: BBI image of 24 hour HUVEC + HUVMSC on ePTFE



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x100

1 mm

Figure 40: SEM at 100x showing sporadic cell clumps and overall bare scaffold

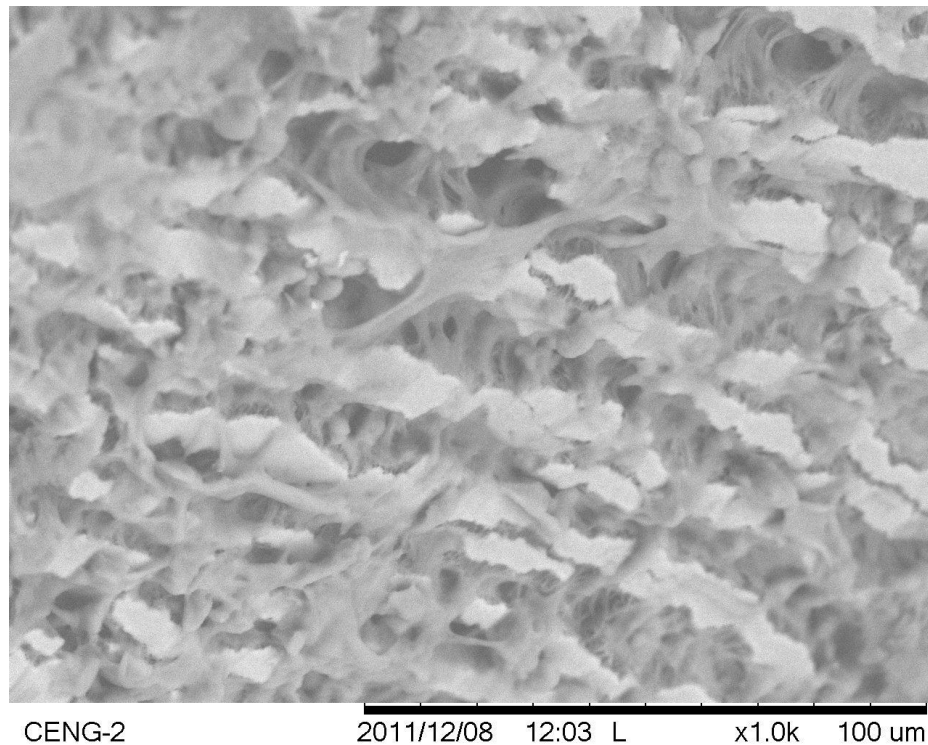


Figure 41: SEM at 1000x showing individual cells covering the ridges of the ePTFE scaffold

Set-up #5

A total of 4 5.5cm x 3.5mm ePTFE scaffolds were dual sodded with HUVEC's and HUVSMC's in the fifth set-up. Initially, there were to be two vessels run for 24 hours and two vessels run for three days, however because one of the T225's broke during the sodding procedure that vessel was converted to a 24 hour vessel, leaving three 24 hour vessels and only one three-day vessel. The first centimeter of the proximal end of each scaffold was imaged with BBI and SEM, and the remaining scaffold was used for RNA isolation. The BBI and SEM results can be seen in Figures 42-49, with the three-day vessel showing the most positive cell deposition and coverage with a similar qualitative result as the dual sodded vessel from set-up #4. This is encouraging, as both set-ups used a similar number of sodded cells, but the surface areas of the vessels seen below are almost double that of the surface area from the fourth set-up.



Figure 42: 3 day bottom 10x

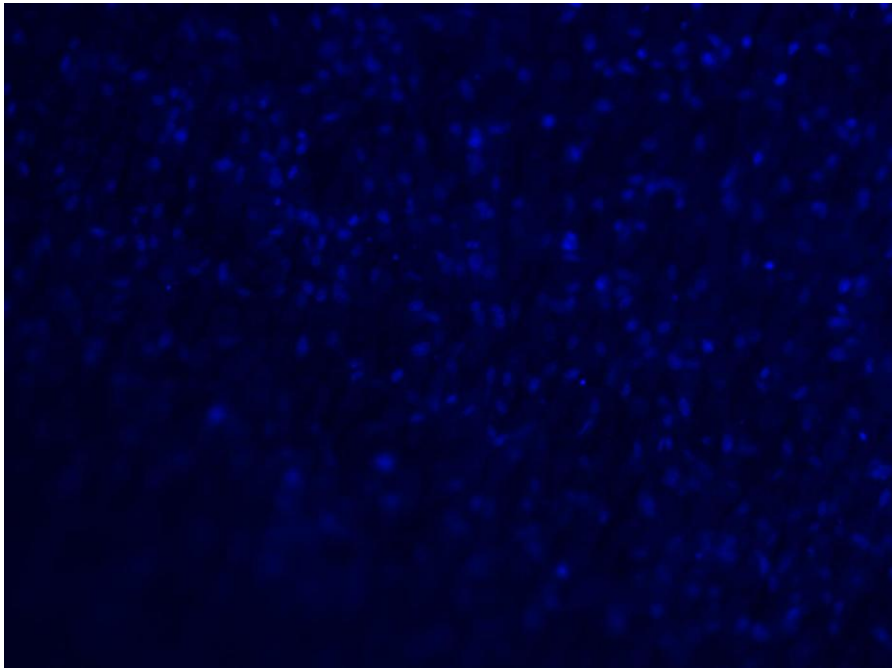


Figure 43: 3 day bottom 10x

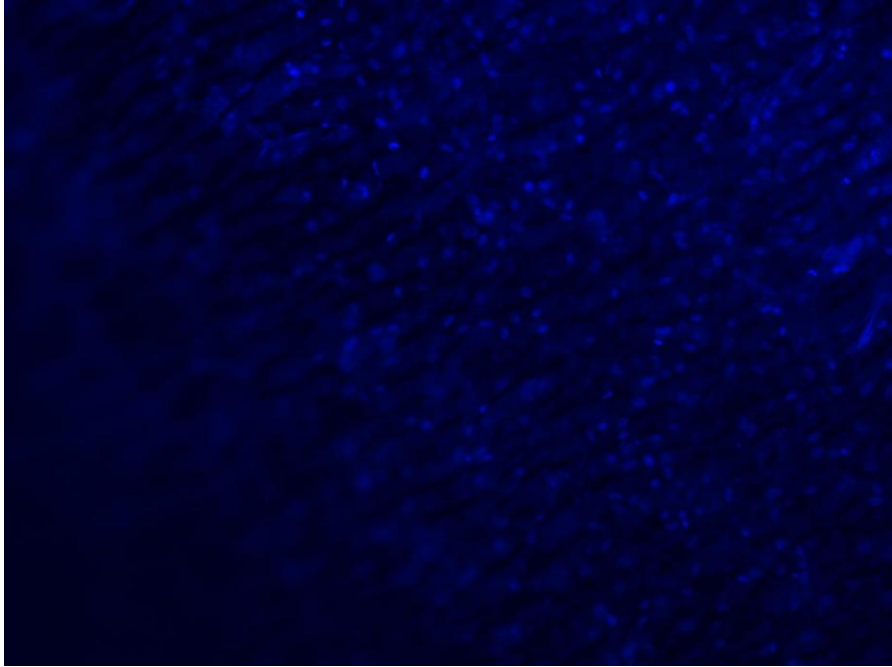


Figure 44: 1 day bottom 10x

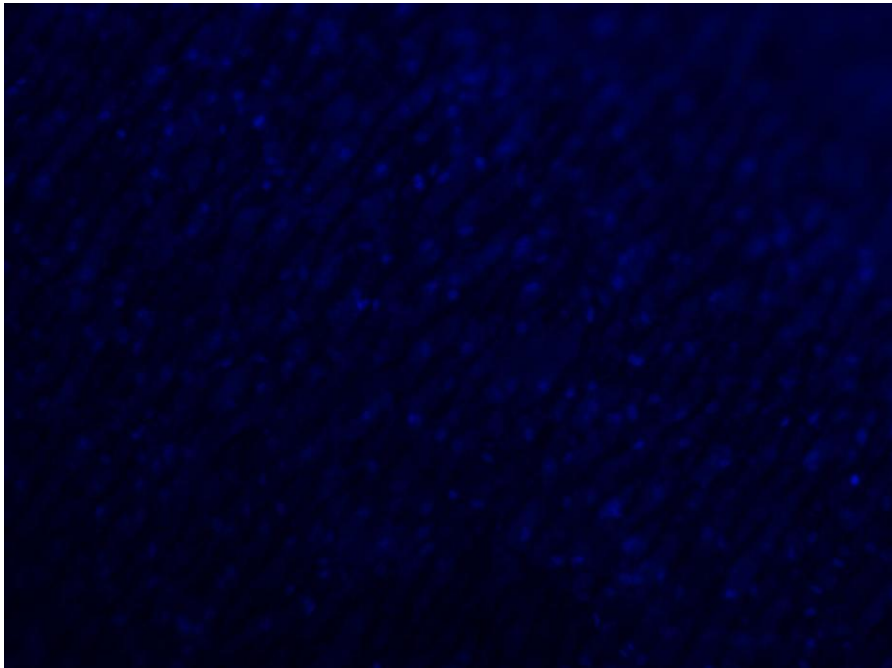


Figure 45: 1 day bottom 10x

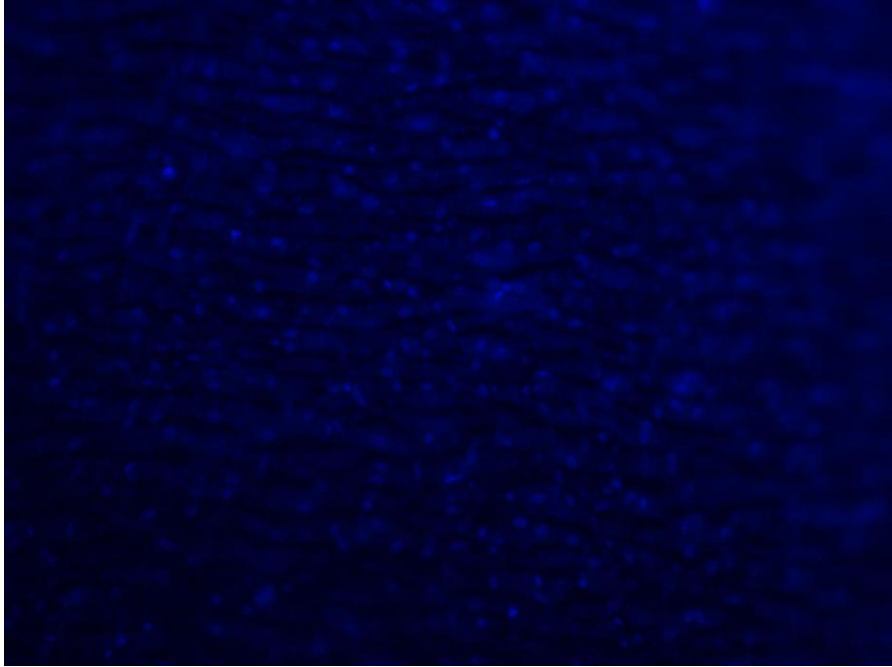


Figure 46: 1 day top 10x

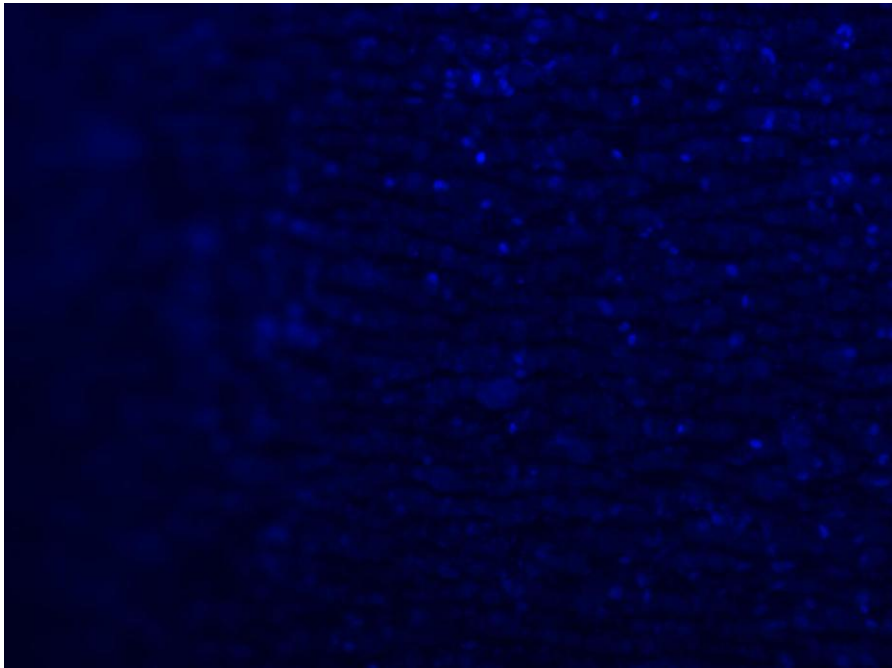


Figure 47: 1 day top 10x

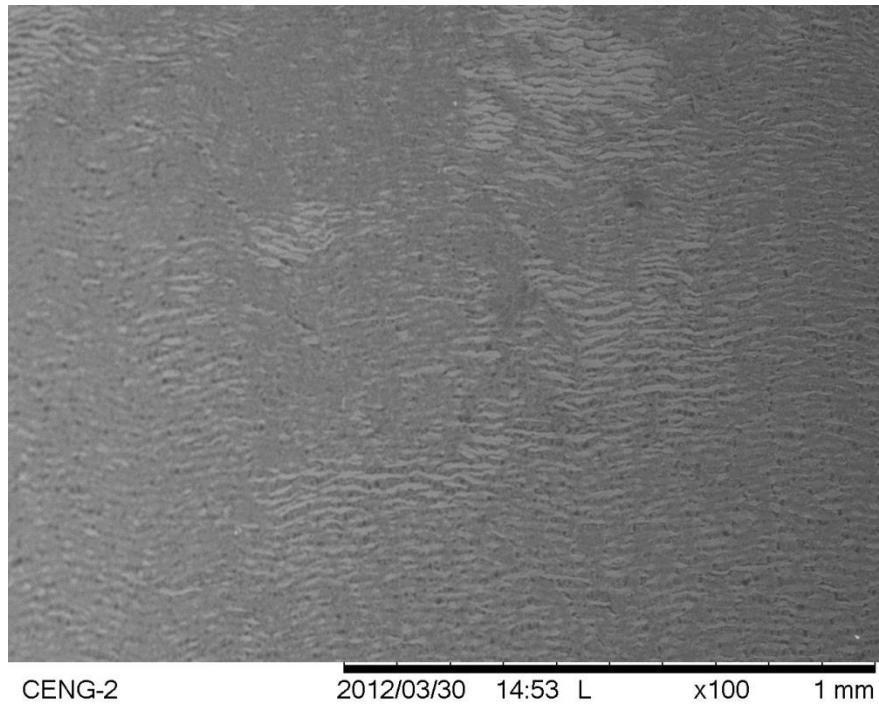


Figure 48: 1 day top 100x

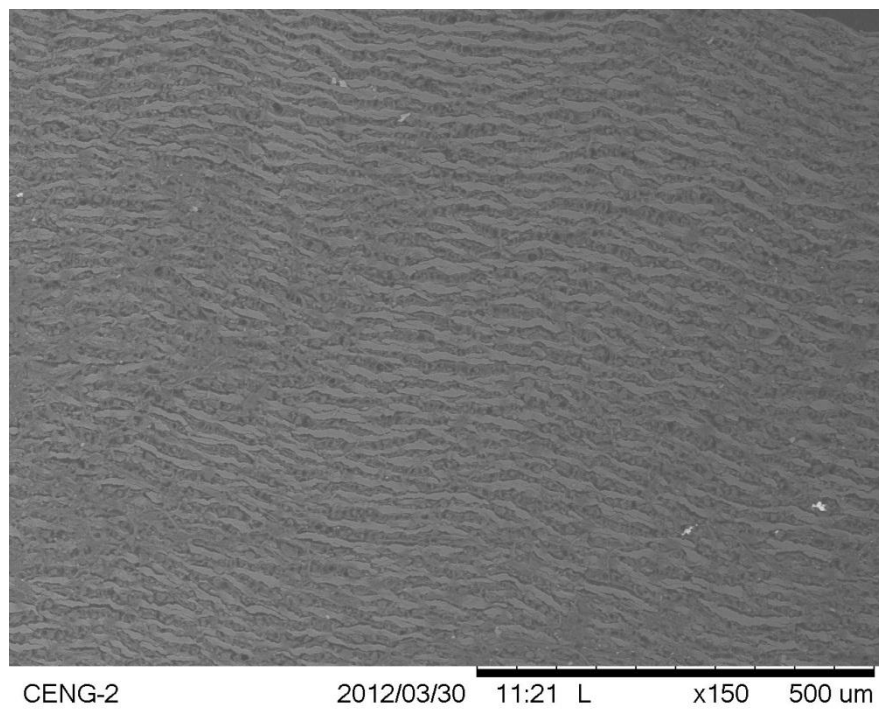


Figure 49: 3 day bottom 150x

Isolation of RNA from these four scaffolds was highly variable. The three day vessel (5A), which had the most positive BBI results, yielded a similar amount of RNA as the two least-covered one day vessels (5C and 5D). The most positive one day scaffold (5B) yielded enough RNA for qPCR, though the spectrophotometry data suggests that the quality of the RNA is questionable. The specific results of the RNA isolation can be found in Table 2. It was noted during the initial cell-lysing step that a significant amount of cell culture media was leached from the ePTFE scaffolds into the lysing buffer, which would severely limit the effectiveness of the lysing buffer in breaking up the cells and freeing the RNA for isolation. These results showed conclusively that this issue as well as the low seeding efficiency must be addressed in order to successfully isolate RNA for qPCR from ePTFE scaffolds.

Set-up #6

The sixth and final set-up also employed 5.5cm x 3.5mm scaffolds. Two scaffolds were taken down and fixed at 24 hours, with the remaining two taken down after three days. The PBS rinse step outlined in the ePTFE take down protocol (Appendix C) was implemented in this set-up, otherwise all procedures were similar to the fifth set-up, with SEM and BBI carried out on ~1cm of the proximal end of the vessel with the rest used for gene expression protocol development. SEM and BBI (Figures 50-55) demonstrate high cell coverage and the development of a monolayer (with some bare spots on the top half of the vessels) in vessels cultured for both one (6A, 6C) and three (6B, 6D) days (images representative of both time points).

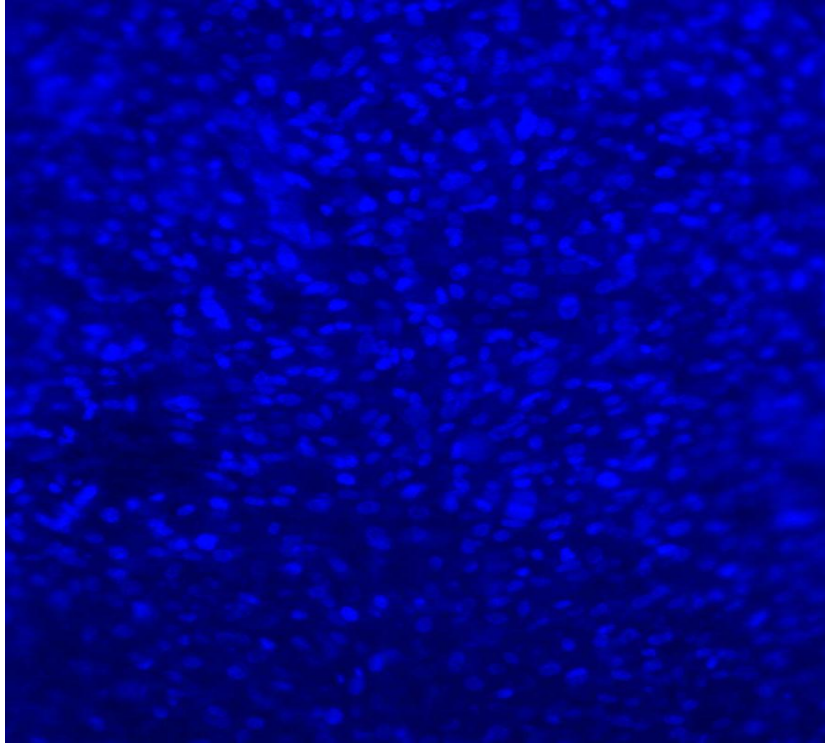


Figure 50: 1-day vessel, bottom half, 10x

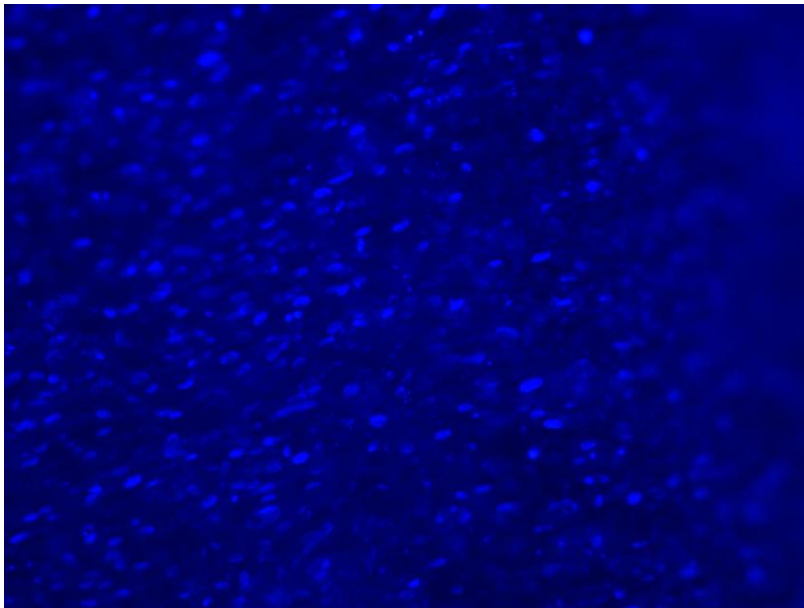


Figure 51: 3-day vessel, bottom half, 10x

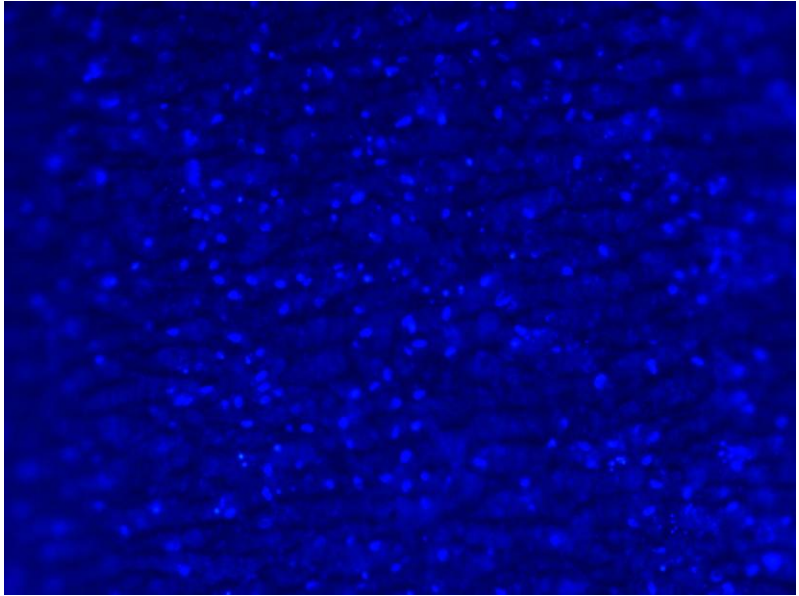


Figure 52: 3-day vessel, top half, 10x

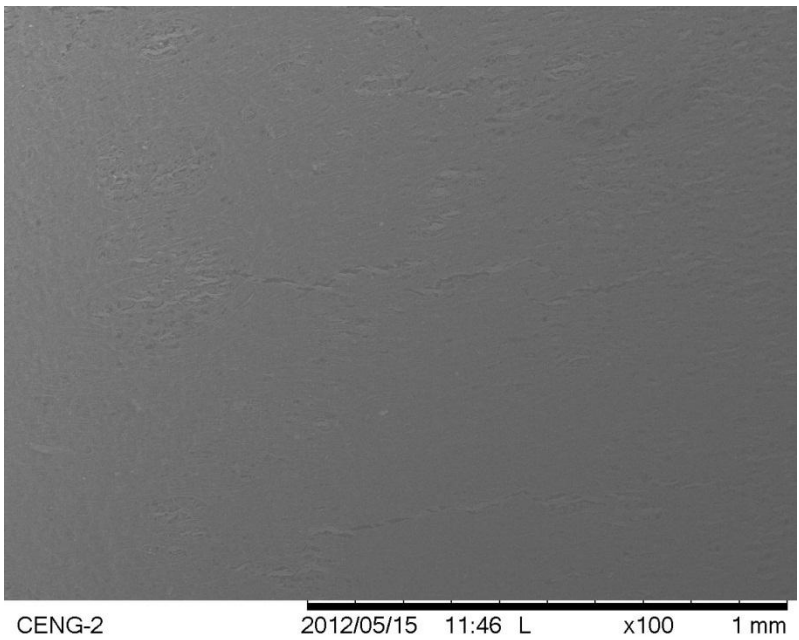


Figure 53: 3-day vessel, bottom half, 100x

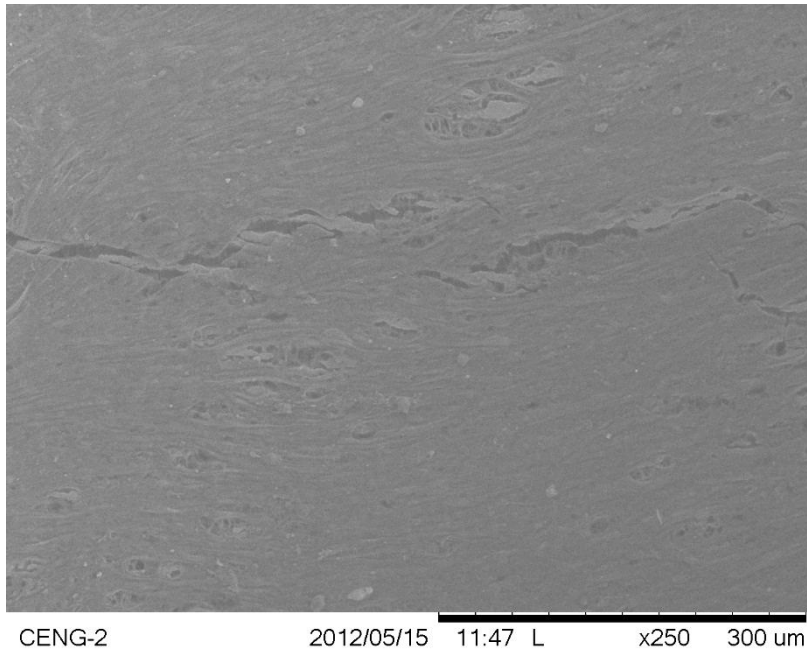


Figure 54: 3-day vessel, bottom half, 250x

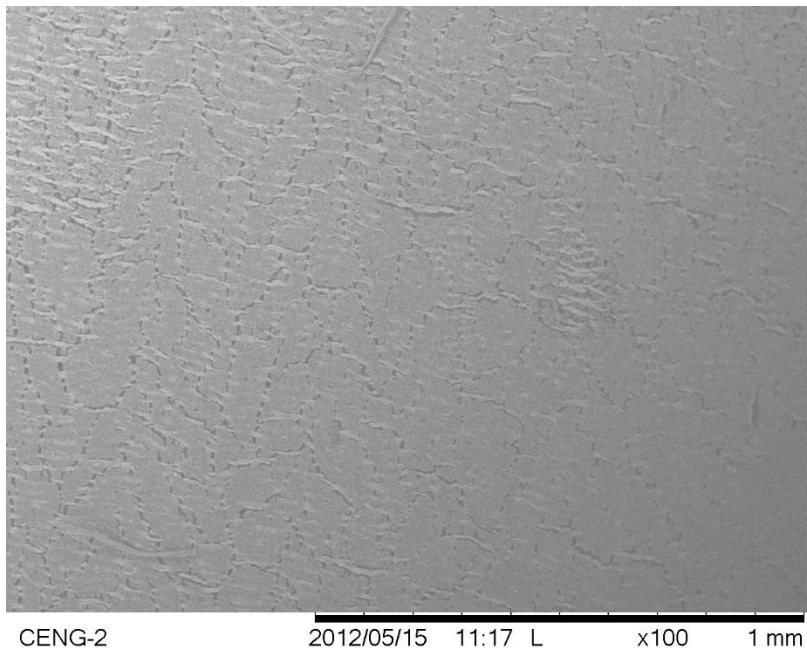


Figure 55: 1-day vessel, top half, 100x

RNA isolation was carried out on these scaffolds with mixed results. Three of the four vessels did not yield enough RNA for gene expression analysis, with only one one-day vessel yielding

sufficient RNA. After comparison of imaging and RNA yield results, the reason for this discrepancy remains unknown.

Table 2: RNA Isolation from ePTFE

Sample	Concentration (ng/uL)	260/280	260/230
1A	6.8	N/A	N/A
1B	11.8	1.74	.54
2A	5.2	2.08	.18
3A	62.5	1.99	1.42
3B	8.0	1.78	.33
5A	47.1	2.73	.78
5B	312.9	1.73	1.41
5C	71.0	2.56	1.82
5D	37.3	3.09	3.47
6A	285.1	2.09	1.48
6B	66.1	2.09	.16
6C	121.3	2.04	1.48
6D	60.8	1.99	1.00

qPCR Results

The primary success of this project was the establishment of a protocol to determine the gene expression of a BVM through qPCR. Although data was gathered, there is a need for more runs to determine significance and for further analysis.

The graph below depict a typical qPCR amplification plot (Figure 56), where the amount of RNA present in the sample is quantified by looking at the cycle where amplification occurred most rapidly. This number is then compared to the reference gene (GAPDH), which should theoretically be expressed at equal levels in all experiments. By normalizing expression to the reference gene, it is possible to compare gene expression across multiple experimental runs.

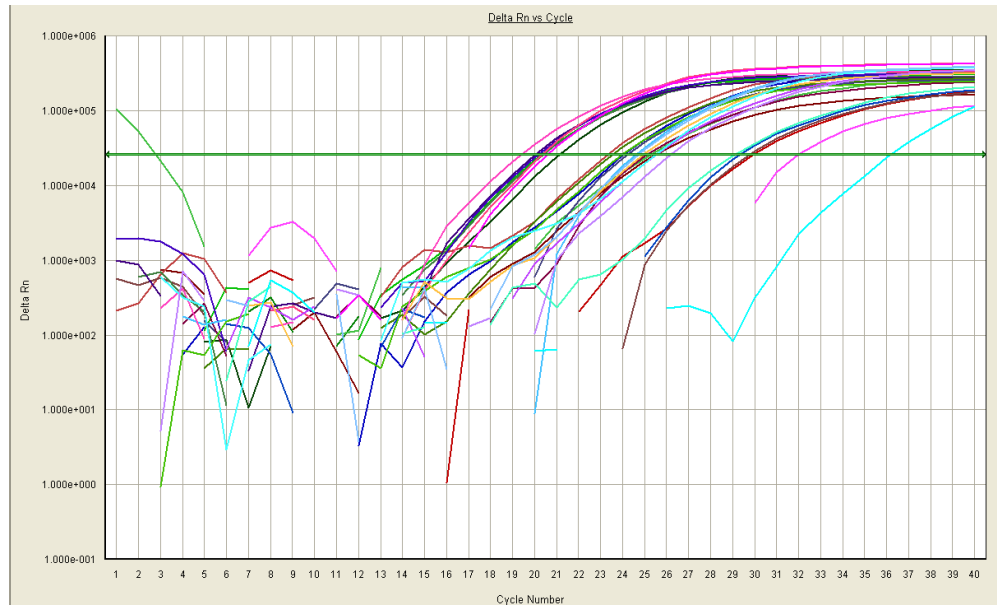


Figure 56: Example Image of Amplification Plots obtained from qPCR

Initial qPCR Attempt

Results from the first qPCR attempt were not optimal. As seen in a representative amplification plot below (Figure 57), samples did not amplify as expected, nor were they consistent between the three wells of each primer.

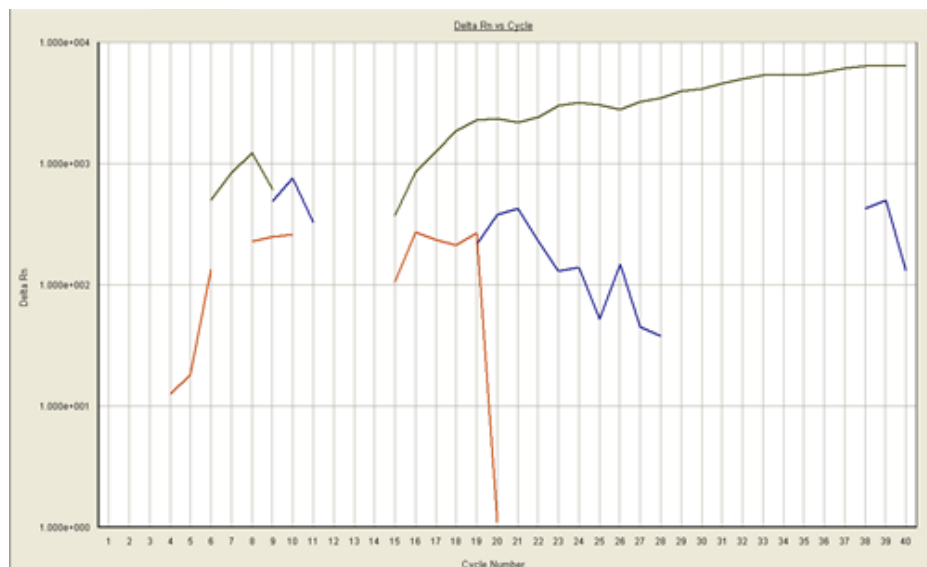


Figure 57: Representative Amplification Plot for a single Primer for the first qPCR attempt

Primer Verification

After the first round of qPCR was unsuccessful, it was necessary to assess the accuracy and efficacy of the primers. To do this, normal PCR was carried out using the primers and sample, and the results were run on a gel as seen in Figure 58. The six bright bands of the same size correspond to the designed lengths of the PCR products, which are about 200 base pairs each. The gel confirmed that the primers produce PCR products of the proper length, and the lack of smearing suggests that the primers are binding only to the target sequences. By verifying that the primers function as designed, it is possible to conclude that any failure in the qPCR reaction is not due to improper primer design.

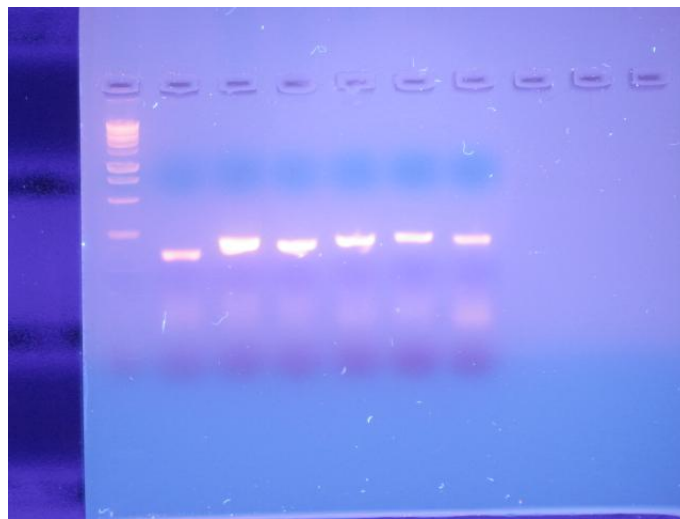


Figure 58: The bright bands at ~ 200bp represent the PCR products generated using the given primers and the lack of smearing suggests specific binding, which combined verify that the primers are operating according to their design.

qPCR Data

Once it was determined that too low a concentration of primers was used for the initial qPCR attempts, following attempts showed consistent amplification curves among primers and samples, as well as minimal amplification from non template controls. qPCR was successfully performed on dual-sodded BVMs of PLGA (Figure 59) and ePTFE (Figure 60). Only two ePTFE BVMs had sufficient RNA for gene expression analysis (5B and 6A). It was found that, though

the quality of the RNA was suboptimal, both samples were successfully used to generate qPCR data, indicating that the protocol was successful on ePTFE BVMs. Representative results confirming the success of the protocol can be seen in Figure 60.

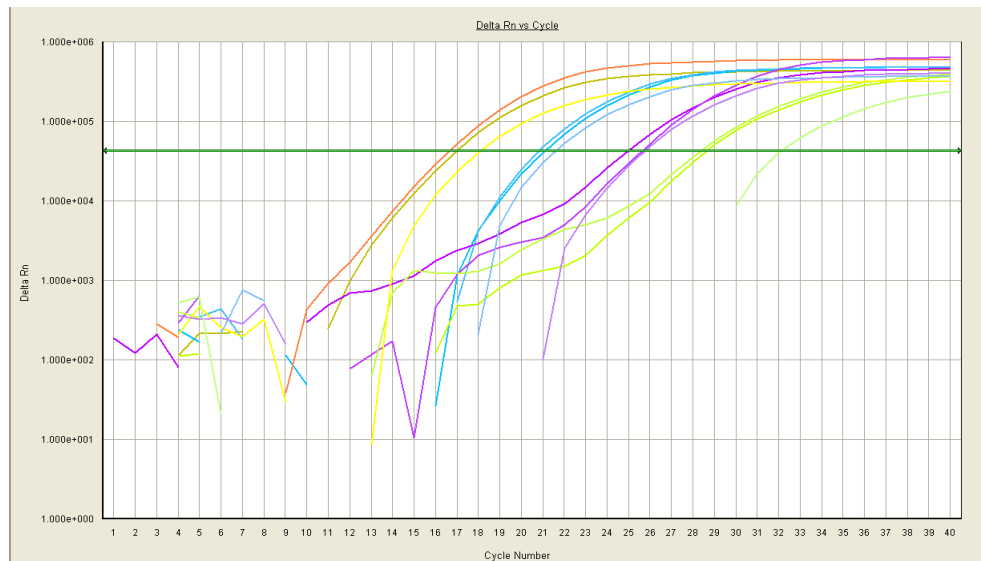


Figure 59: Amplification Plot of PLGA BVM Samples

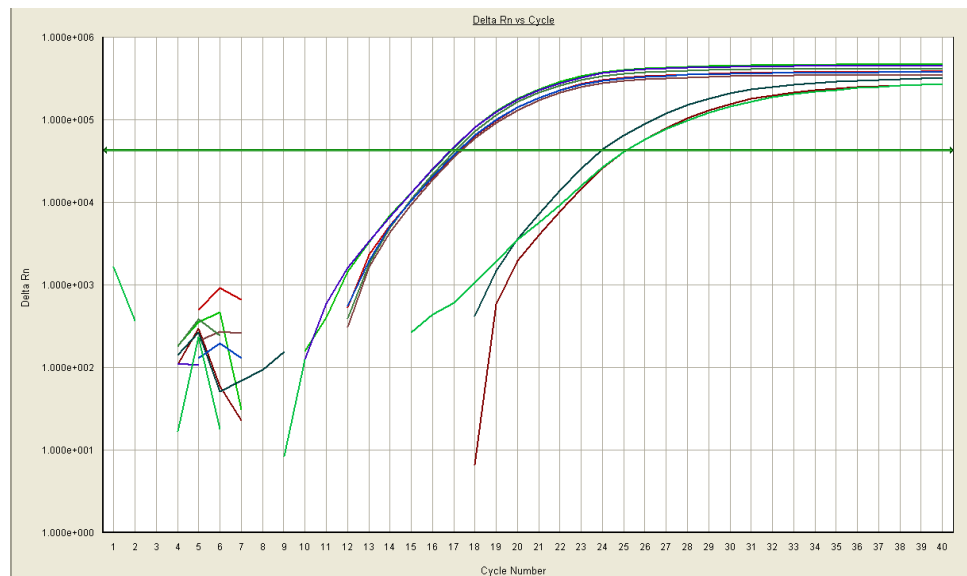


Figure 60: Amplification Plot of ePTFE BVM Samples

Quantified results from all qPCR runs can be seen in Table 3 and Figure BLEH below. However, due to a lack of quantity of RNA and amount reagents, some samples could not be run with all primers, represented by an NA in Table 4 below.

Table 4: qPCR Gene Expression Cycle Threshold (Ct) Results

	ICAM	Ki67	PECAM	Bax
ePTFE 1-day	-.32177	-17.2402	7.5005	NA
Cultured Cells	5.976	5.50604	5.47284	NA
ePTFE 1-day	-.8533	6.57637	4.1065	NA
PLGA 3-day	3.89517	8.19733	12.46027	NA
PLGA 1-day	-13.4488	1.3047	1.74324	NA
PLGA 3-day	-13.8287	4.85533	.9575	NA
PLGA 1-day	3.8365	5.22753	NA	9.4061
PLGA 1-day	NA	2.94675	4.54385	NA
PLGA 3-day	5.5858	6.1056	12.6577	937479
PLGA 3-day	NA	4.21095	5.40135	15.76965

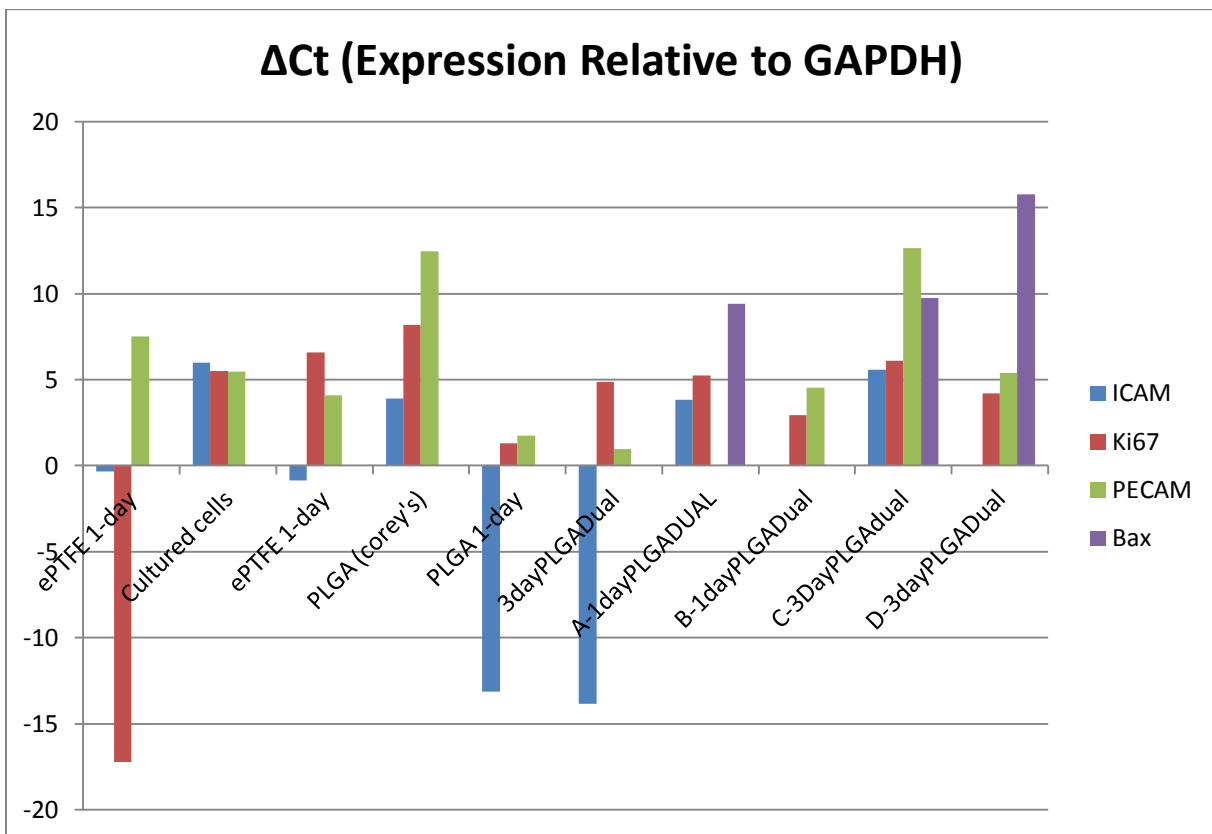


Figure 61: Delta Ct Expression Relative to GAPDH for all Samples

CHAPTER 4: Discussion and Conclusions

The goal of this project was to develop the BVM stent testing platform and to develop a protocol for gene expression analysis that would ultimately allow the quantification of the performance of various stent designs tested in the BVM system. Both PLGA and ePTFE BVM protocols were developed such that nearly 100% confluent cell linings were established, and RNA isolation and gene expression analysis could be carried out. Furthermore, data collection on the gene expression in unstented PLGA and ePTFE BVMs was started, with methods for future analysis outlined such that when the necessary remaining data has been collected, quantification of results should be straightforward.

BVM Development

The first phase of the project was developing the protocol for PLGA and refining the protocol for ePTFE such that RNA could be successfully isolated from each material.

PLGA

A protocol for PLGA was developed based on an existing protocol for ePTFE using HUVECs.

The protocol was modified to change the method of sterilization from autoclaving to a 30 minute 70% Ethanol sterilization. For PLGA Set Up #1, this method of sterilization was sufficient to not result in any contamination, however the low cell adhesion needed to be improved. In order to increase adhesion, modifications made to the original protocol to make the flow increase regime more gradual. Although this change in flow increased cell adhesion (Set Up #2), confluency remained much lower than was desired.

One of the larger changes made to the protocol was the addition of a new cell type, HUVMSCs. Due to their place in a native vessel, it was determined that smooth muscle cells had the potential to not only increase the physiologic relevance of the PLGA BVM but also increase endothelial cell adhesion. Set Ups #3-5 focused on integrating a second cell type into the BVM. This

integration of HUVSMCs increased the confluency of BVMs to nearly 100% while maintaining the presence of both cell types. Over the course of 5 set ups, the PLGA BVM protocol was modified from a non-confluent single cell protocol to a confluent dual cell protocol. Both the original and final protocols can be found in Appendix A.

ePTFE

For ePTFE, the original protocol, which involved single sodding HUVECs, was modified to include the addition of HUVSMCs while maintaining the original conditioning steps. Initial experiments (Set Up #1 and #2) showed extremely low cell adhesion and coverage when sodding with only a single cell type, similar to early results on PLGA. The addition of HUVSMCs served two purposes: to increase cell adhesion by adding a cell type that endothelial cells naturally adhere to, and to increase the overall physiological relevance of the system by introducing a medial smooth muscle layer into the construct. HUVSMCs had already been shown to increase cell adhesion on PLGA, and subsequent experiments employing HUVSMCs on ePTFE BVMs showed similar increases in cell adhesion and coverage with scaffolds reaching near 100% confluence in later experiments (see Set Up #6). In the later experiments, the protocol was preserved through the dynamic conditioning steps, with the cell sodding portion of the protocol replaced by the cell sodding protocol developed for PLGA. The full modified protocol can be found in Appendix A.

The other major change to the ePTFE protocol involved the addition of a gene expression specific take-down and fixation protocol (Appendix C). After initial attempts to isolate RNA from ePTFE BVMs were unsuccessful, new fixation and processing techniques were attempted. The first modification tested was a grinding step in which bare ePTFE was cooled in liquid nitrogen and then ground using a tissue homogenizer, in the hope that the cells would be freed

from the scaffold mechanically. This was a complete failure, as the ePTFE scaffold simply smashed down and did not show any signs of deconstruction. The second modification, which ultimately proved successful, was the addition of a PBS rinse step before fixation. The process employed a luer lock syringe attached to the inlet port of the bioreactor which was used to flush cell culture media from the scaffold. By removing the cell culture media, which was known to inhibit the cell lysing buffer used in the first step of RNA isolation, the sample was cleared of inhibitory proteins and the isolation of RNA from the ePTFE scaffolds was successfully accomplished.

In conclusion, the protocols for constructing both PLGA and ePTFE BVMs were developed and refined to the point that physiologically relevant, confluent cellular layers were developed, and further protocols were developed in order to prepare those samples for successful gene expression analysis.

Gene Expression Protocol Development

Using commercially available kits (for RNA isolation, reverse transcription and qPCR), a gene expression protocol was successfully developed. Overall the protocols supplied with the kits were followed, with a few minor variations. These included the rinse steps added before RNA isolation, a five minute incubation during the initial lysing step of RNA isolation, and employing a master mix for qPCR with only one half the recommended amount of SYBR green dye. The exponential growth curves obtained from qPCR analysis were highly consistent, indicating a high level of repeatability and reliability. One minor issue that remains to be completely solved is the evidence of contamination in the non-template controls, where the non-template controls amplify along with the samples, though at much later time points. Generally, contamination of the non-template control indicates DNA contamination that could compromise the validity of the

results, especially if the amplification occurs at time points close to the sample. The non-template controls in this study did amplify (only approximately half of the non-template controls showed signs of amplification), but because it occurred so much later than the sample it is reasonable to conclude that the non-template amplification was due to minor flaws in the preparation technique and not large scale contamination that would skew the results of the analysis overall.

Because the protocol was successful, initial data collection on the unstented BVMs was possible. Using the Ct values obtained from the 7500 Fast System SDS Software, we were able to determine the ΔC_t value for a limited number of the genes of interest for BVMs cultivated for both one and three day time points. However, due to a limited number of samples and the nature of gene expression analysis, we were unable to draw any conclusions about the levels of gene expression in the BVMs, as more data points are required for almost all genes, and there is no control to which to relate the obtained data.

In conclusion, the gene expression protocol was successfully developed and is now ready to be employed on both stented and unstented BVMs.

Future Work

The next immediate step for this project is the full characterization of the gene expression in unstented BVMs, which, once accomplished, will be able to serve as a control for all subsequent stented BVMs. Because gene expression analysis relies on relative and not absolute values, it is impossible to draw any conclusions about gene expression before a given gene has been fully characterized for at least two different treatment types (i.e. unstented and stented BVMs).

Another possible control could be the analysis of gene expression of cells in static culture, which

if quantified would allow tracking of changes in gene expression from culture to unstented BVM to stented BVM.

Once the appropriate controls have been quantified and analyzed, the next step will be to begin to test stents in the BVMs and execute gene expression analysis on stented BVMs to determine changes in gene expression due to stenting. For this process, there will be two potential points of comparison: the initial, unstented control and the stent in question, and the stent in question compared to another stent. It is the second comparison that is perhaps the most useful potential tool of this system, where stents can be tested side by side and their performance can be quantitatively compared via qPCR. The implementation of the gene expression protocol developed in this report is perhaps the most powerful tool available for evaluating stent performance in the BVM system, and brings the BVM as a testing platform much closer to the realization of an in vitro testing platform that will provide some quantitative, predictive data that can be used to evaluate potential stent designs before moving into more costly and ethically challenging animal studies.

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Appendix A: BVM Protocols

Date _____	Blood Vessel Mimic Protocol (ePTFE)	Initials _____
Study Information		
Study Title: _____		
Purpose: _____		
Material: _____		
Diameter: _____		
Length: _____		
Cell Type: _____		
Prep 1 week prior		
____ 1.	Gas sterilize biochambers (with extra piece of tubing) and 2-port reservoirs	____ 15. Record BVM numbers (or chamber ID):
____ 2.	Determine target number of cells and passage schedule, then thaw cells	
____ 3.	Cut grafts, mount on fittings, and suture	
____ 4.	Autoclave grafts, flasks, and forceps	
Prep the day before		
____ 5.	Make media:	
a.	Bioreactor Media (Human Complete w/o ECGS w/ antibiotics)	
b.	Conditioning Media (1:6 solution of FBS:M199 + antibiotics)	
____ 6.	Denude vessels	
a.	15 min 70% EtOH	
b.	15 min 100% EtOH	
c.	Place vessels in conditioning media (at least 24 hrs before set-up)	
Set-up day: BVM conditioning		
____ 7.	Fill chamber with Bioreactor media (Be sure tubes are closed!)	
____ 8.	Insert sterile grafts into biochambers. And place lid on chamber. ADD STOP CAP TO INLET.	
____ 9.	Using a syringe, flush lumen with Conditioning Media to prime graft	
a.	Clamp lumen and continue to prime graft	
b.	Repeat for all vessels	
____ 10.	Prime 2-port reservoirs with Conditioning Media	
a.	Attach primed biochamber to 2-port reservoir and condition graft for 20 min	
b.	Flow through lumen first to remove air, then clamp lumen and condition transmurally on 150 rpm setting	
____ 11.	Leave primed biochambers in large incubator until ready for seeding step	
Set-up day: BVM seeding		
____ 12.	Prime 2-port reservoirs with HUVEC and HUVEC media (3:1)	
a.	Prepare one for each vessel	
b.	Be sure that drip is visible and outlet is submerged	
c.	Clamp tubing and leave in big incubator	
____ 13.	Take corresponding number of primed bioreactors and reservoirs to hood	
____ 14.	Attach outlet of reservoir to inlet stopcock of biochamber	
a.	Leave reservoir inlet unattached and biochamber outlet facing trough	
____ 16.	Harvest HUVECs	
a.	Apply Trypsin, deactivate with media	
b.	Take 100 μ L from total _____ mL cells	
	Counts:	
____ 17.	X = _____ x 2000 x (cell/mL x .10)	
	total number of cells = _____	
____ 18.	Pellet cell suspension (on 4 for 4 min)	
____ 19.	Resuspend in 3 ml Bioreactor Media per vessel	
____ 20.	Seed each graft with _____ mL cell solution	
a.	Cells per graft = _____	
b.	Seeding density = _____ cells/cm ²	
____ 21.	Chase with 6 mL Bioreactor Media	
____ 22.	Attach biochamber outlet to reservoir inlet	
____ 23.	Bring BVMs to large incubator	
____ 24.	Place on small WM pump - leave lumen clamped!!	
____ 25.	Begin transmural flow at 10 rpm, and maintain for 10 mins	
a.	Started on pump at: _____ (time)	
____ 26.	Turn off pump and allow cells to rest for 1-3 hrs.	
____ 27.	Repeat steps 18-27 for HUVECs.	
____ 28.	Turn off pump and allow to rest for 3 hours to overnight.	
____ 29.	Unclamp lumen and maintain 15 rpm luminal flow for 3-6 hours	
____ 30.	Increase flow to 25 rpm leave for 3 hours.	
____ 31.	Increase flow to 45. leave for 3 hours.	
The next day and beyond		
____ 32.	Increase flow by 10-15 rpm at a time to reach 90 rpm by the end of the day	
____ 33.	BVM maintenance: replace media reservoirs every 3 rd day	
a.	Check CO ₂ !	

Date _____ Blood Vessel Mimic Protocol (PLGA single sod) Initials _____

Study Information

Study Title: _____

Purpose: _____

Material: _____

Diameter: _____

Length: _____

Cell Type: _____

Prep 1 week prior

- ____ 1. Gas sterilize biochambers (with extra piece of tubing) and 2-port reservoirs
- ____ 2. Determine target number of cells and passage schedule, then thaw cells
- ____ 3. Cut grafts, mount on fittings, and suture
- ____ 4. Autoclave grafts, forceps, and stop caps

Prep the day before

- ____ 5. Make media:
 - a. Bioreactor Media (Human Complete w/o ECGS w/ antibiotics)
 - b. Conditioning Media (1:6 solution of FBS:M199 + antibiotics)

Set-up day: BVM conditioning

- ____ 6. Sterilize grafts (using 70%)
 - a. 30 min 70% EtOH
 - b. Using syringe and cap flush luminal and transmurally with PBS
 - c. Perform same flush procedure with Conditioning Media
- ____ 7. Place small WM pump in hood
- ____ 8. Fill chamber with Bioreactor media (Be sure tubes are closed!)
- ____ 9. Insert sterile grafts into biochambers. And place lid on chamber. ADD STOP CAP TO INLET.
- ____ 10. Prime 2-port reservoirs with Conditioning Media
- ____ 11. Attach primed biochamber to 2-port reservoir and condition graft for 6 hours-overnight
 - a. Flow through lumen first to remove air, then clamp lumen and condition transmurally on 150rpm setting
- ____ 12. Leave primed biochambers in large incubator until ready for sodding step

Set-up day: BVM sodding

- ____ 13. Prime 2-port reservoirs with HUVEC Media
 - a. Prepare one for each vessel
 - b. Be sure that drip is visible and outlet is submerged
 - c. Clamp tubing and leave in big incubator
- ____ 14. Take corresponding number of primed biochambers and reservoirs to hood
- ____ 15. Attach outlet of reservoir to inlet stopcock of biochamber
 - a. Leave reservoir inlet unattached and biochamber outlet facing trough

____ 16. Record BVM numbers (or chamber ID):

- ____ 17. Harvest HUVECs
 - a. Apply Trypsin, deactivate with media
 - b. Take 100uL from total _____ mL cells
Counts:

____ 18. $X = \frac{\text{total number of cells}}{\text{cell mL} \times .10}$

- ____ 19. Pellet cell suspension (on 4 for 4 min)
- ____ 20. Resuspend in 3 ml HUVEC Media per vessel
- ____ 21. Sod each graft with 3 mL cell solution
 - a. Cells per graft = _____
 - b. Sodding density = _____ cells/cm²
- ____ 22. Chase with 3 mL HUVEC Media
- ____ 23. Attach biochamber outlet to reservoir inlet
- ____ 24. Bring BVMs to large incubator
- ____ 25. Place on small WM pump - leave lumen clamped!!
- ____ 26. Begin transmural flow at 10rpm, and maintain for 10 mins
 - a. Started on pump at: _____ (time)
- ____ 27. Return to large incubator allow to rest for 6 hours to overnight.
- ____ 28. Unclamp lumen and maintain 15rpm luminal flow 3hrs
- ____ 29. Increase flow to 25rpm leave 3 hrs.
- ____ 30. Increase flow to 55. leave for 3 hours.

The next day and beyond

- ____ 31. Increase flow by 10-15rpm at a time to reach 90rpm by the end of the day
- ____ 32. BVM maintenance: replace media reservoirs every 3rd day
 - a. Check CO₂!

Date _____ Blood Vessel Mimic Protocol (PLGA dual sod) Initials _____

Study Information

Study Title: _____

Purpose: _____

Material: _____

Diameter: _____

Length: _____

Cell Type: _____

Prep 1 week prior

1. Gas sterilize biochambers (with extra piece of tubing) and 2-port reservoirs
2. Determine target number of cells and passage schedule, then thaw cells
3. Cut grafts, mount on fittings, and suture
4. Autoclave grafts, forceps, and stop caps

Prep the day before

5. Make media:
 - a. Bioreactor Media (Human Complete w/o ECGS w/ antibiotics)
 - b. Conditioning Media (1:6 solution of FBS:M199 + antibiotics)

Set-up day: BVM conditioning

6. Sterilize grafts (using 70%)
 - a. 30 min 70% EtOH
 - b. Using syringe and cap flush lumenally and transmurally with PBS
 - c. Perform same flush procedure with Conditioning Media
7. Place small WM pump in hood
8. Fill chamber with Bioreactor media (Be sure tubes are closed!)
9. Insert sterile grafts into biochambers. And place lid on chamber. ADD STOP CAP TO INLET.
10. Prime 2-port reservoirs with Conditioning Media
11. Attach primed biochamber to 2-port reservoir and condition graft for 6 hours-overnight
 - a. Flow through lumen first to remove air, then clamp lumen and condition transmurally on 150rpm setting
12. Leave primed biochambers in large incubator until ready for seeding step

Set-up day: BVM seeding

13. Prime 2-port reservoirs with HUVEC/HUVSMC Media mixed 2:1
 - a. Prepare one for each vessel
 - b. Be sure that drip is visible and outlet is submerged
 - c. Clamp tubing and leave in big incubator
14. Take corresponding number of primed biochambers and reservoirs to hood
15. Attach outlet of reservoir to inlet stopcock of biochamber
 - a. Leave reservoir inlet unattached and biochamber outlet facing trough

16. Record BVM numbers (or chamber ID): _____

17. Harvest HUVECs
 - a. Apply Trypsin, deactivate with media
 - b. Take 100uL from total _____ mL cells
Counts: _____

18. $X = \frac{\text{total number of cells}}{\text{cell mL} \times .10}$

19. Pellet cell suspension (on 4 for 4 min)
20. Resuspend in 3 mL Cell Media per vessel
21. Seed each graft with 3 mL cell solution
 - a. Cells per graft = _____
 - b. Seeding density = _____ cells/cm²
22. Chase with 3 mL Cell Media
23. Attach biochamber outlet to reservoir inlet
24. Bring BVMs to large incubator
25. Place on small WM pump - leave lumen clamped!!
26. Begin transmural flow at 10rpm, and maintain for 10 mins
 - a. Started on pump at: _____ (time)
27. Turn off pump and allow cells to rest for 1-3 hrs.
28. Repeat steps 18-27 for HUVSMC cells.
29. Return to large incubator allow to rest for 6 hours to overnight.
30. Unc clamp lumen and maintain 15rpm luminal flow 3hrs
31. Increase flow to 25rpm leave 3 hrs.
32. Increase flow to 55. leave for 3 hours.



The next day and beyond

33. Increase flow by 10-15rpm at a time to reach 90rpm by the end of the day
34. BVM maintenance: replace media reservoirs every 3rd day
 - a. Check CO₂!

Appendix B: Imaging Protocols

Procedure for Bisbenzimidide (BBI) Evaluation of Cell-Sodded Scaffolds

Written by: R. Dalton Chavez

1. Wrap nonsterile 15-mL conicals in foil. Thoroughly label conicals for all samples.
2. Make BBI solution. Always keep stock solution wrapped in foil!
 - a. Use small tube of stock solution and dilute 1:1000 with milli-Q water (10 μ L stock solution in 10 mL water). Use pipette aid from room 209 hood for milli-Q water; spray pipette aid before placing back in hood.
 - b. Mix by inverting.
3. Put on safety glasses. Cut samples with blade or scissors, being careful to not disrupt cell lining inside lumen (squeezing, touching, and scraping can all disrupt lining and ruin experiment).
4. Use washed forceps to place samples in corresponding 15-mL conicals that contain BBI solution. Carefully keep track of samples. Leave foil on conicals.
 - a. Let samples soak for at least 15 minutes (longer is better).
5. Put away extra stock solution. Clean up preparation area.
6. With permission or help from Dr. Cardinal, use fluorescent microscope to obtain *en face* images. Take forceps to use at microscope. See figure 1 for pictures of steps below.
 - a. Log into notebook (fluorescence; initials; date; time and lamp hours).
 - b. Turn turret to setting 4.
 - c. Turn on Olympus lamp (green switch).
 - d. Turn on Optiscan wheels (black switch).
 - e. Turn on camera (black switch).
 - f. Set filter wheel 1 to 1, and set filter wheel 2 to 1.
 - g. Open shutter: .
 - h. Set Prior keypad to shutter S1.
 - i. Dial objectives to desired magnification (typically 4x and 10x for BBI images).
 - j. Set thin bar to icon of eye and camera.
 - k. Sign into computer by clicking Kristen's account (password can be obtained from Kristen).
 - l. Click QCapture Pro (on desktop).
 - m. Click camera icon at top of QCapture Pro window: .
 - n. Place slide on microscope. Place sample on slide.
 - o. See figure 2.
 - p. Manually adjust microscope to clarify image.
 - q. If scaffold is too wet, carefully blot end of scaffold on Kim wipe.
 - r. Take pictures. Save pictures if desired (labeled with sample info, initials, and magnification).
 - s. Quit QCapture Pro. Log out of computer.
 - t. Shut down microscope by switching off Olympus lamp, switching off Optiscan wheels, switching off camera (on top of microscope), closing shutter, and setting thin bar to icon of eye.
 - u. Log out of notebook.

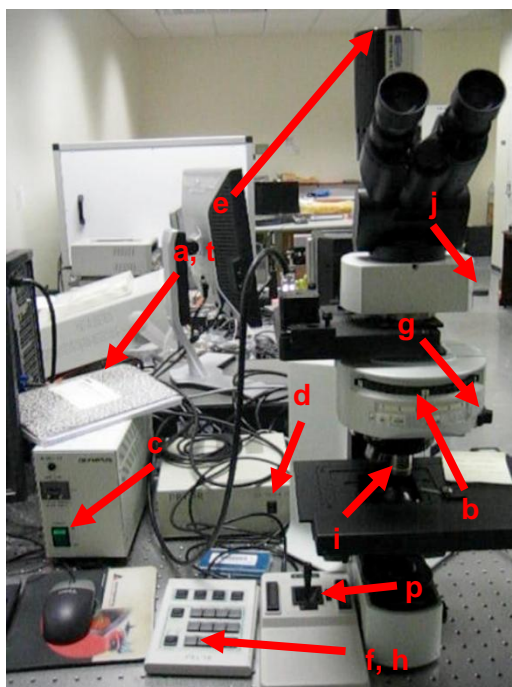


Figure 1: Microscope anatomy. Letters correspond to step 6 in protocol.

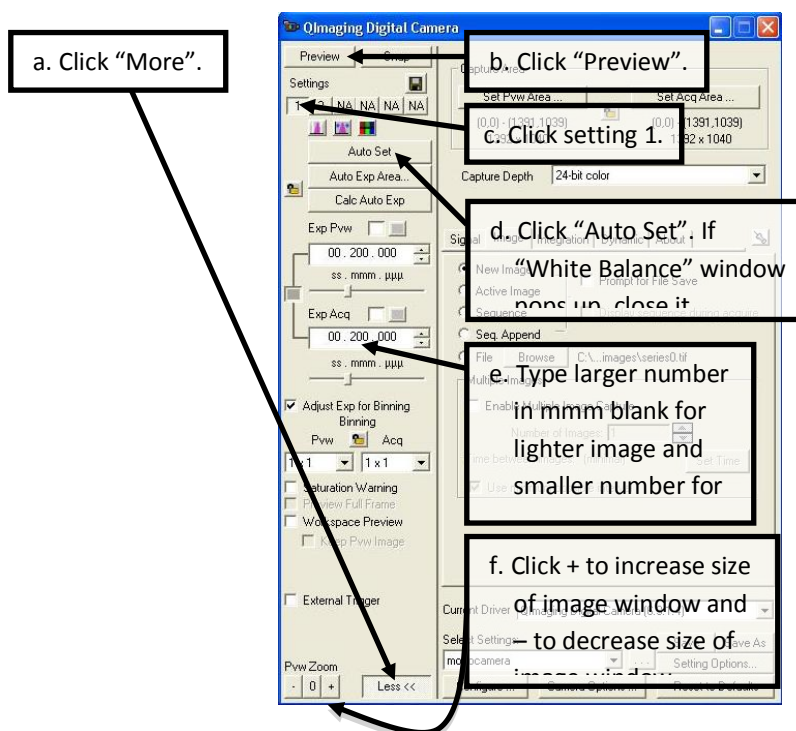


Figure 2: QCapture Pro control panel.

Sample Preparation for Scanning Electron Microscopy

Materials:

6 Coverslips with cells on them
Distilled water Histochoice
100% Ethanol 50 mL conical
tubes for waste

Note: for all steps, do not pipette any solutions directly on to the cells as this will dislodge cells.

1. Remove cell media from each well.
 2. Remove the growth media and replace with 3 mL of fixative (Histochoice) for each well of a six well plate. Cover each coverslip with 3 mL of solution for 30 minutes at room temperature. Cover six well plate with lid.
 3. Remove histochoice and put into 50 mL conical tube as liquid waste. Clearly label your waste containers.
 4. Gently wash the cells with distilled water. Repeat 4 times.
 5. Prepare the following solutions by diluting with distilled water: 25% ethanol, 50% ethanol, 70% ethanol, 90% ethanol, and 100% ethanol. You will be using 1 mL of solution per coverslip.
 6. Dehydrate the cells in the following steps:
 - 25% ethanol for 5 minutes
 - 50% ethanol for 5 minutes
 - 70% ethanol for 5 minutes
 - 95% ethanol for 5 minutes
 - 100% ethanol for 5 minutes (2 times)
- Due to the fragility of the coverslips, you will be performing the dehydration steps within the 6 well plate. Completely remove the solution from the previous step.
7. Remove coverslips from solution and air dry. Mount each coverslip on a separate glass slide using double stick tape. Ensure the coverslips are oriented with the cells exposed to air. Transfer slides to petri dishes for transport to SEM microscope. Make sure coverslips are completely dry before imaging.
 8. You are now ready to image your cells.

The Use of Cell Trackers for Dual Sodding

Purpose:

The overarching purpose of this protocol is to develop a method to pressure sod two different cell types onto a scaffold for the BVM using two different fluorescent cell trackers. The concept of the cell tracker is to visually monitor a dyed cell as it migrates, divides or combines with other cells types using a wide field fluorescent microscope. This protocol aims to provide successful dual sodding (ie. pressure sodding two different cell types onto the vessel).

Materials

- 5-20 μ L pipette and sterile head (usually in the hood in 209)
- 3 ml Syringe with Needle tip
- DMSO 100mL bottle from the corner cupboard (Sigma-Aldrich) catalog number 276855 (anhydrous)
- Cell tracker Red CMPX (Invitrogen; Carlsbad, CA) catalog number C34522
- Cell Tracker Green CMFDA (5- Chloromethyl Fluorescein Diacetate; Invitrogen; Carlsbad, CA) catalog number C 7025
- Sterile Serum-free Media (for whatever cell type you are using)
- 1 – 25 ml pipettes (the regular ones we use)

Methods

Basic Methods

The cell tracker dye was made following the manufacturers instructions. The stock solution was created by adding high-quality DMSO to the lyophilized dye product. 10.8 μ L of DMSO was added to the 50 μ g of cell tracker to produce a 10mM concentration of **stock solution**. Then dilute the stock solution with serum free media to a concentration of 0.5 to 20 μ M (this work shows a 5 μ M concentration, but other concentrations are characterized below in the graph), this is the **working solution**. Differences in concentration depend upon the specific cell type and application of the cells. Higher concentrations are used for cells which will go through several population doublings or for longer durations prior to imaging. Lower concentrations of the working solutions can be utilized for less proliferative cells or for short term experiments. Two cell trackers were utilized in this methodology: Cell Tracker Green CMFDA

(5- Chloromethyl Fluorescein Diacetate; Invitrogen; Carlsbad, CA) catalog number C 7025 and Cell Tracker Red CMPX (Invitrogen; Carlsbad, CA) catalog number C34522.

Once the cells have been cultured to the desired confluency the actual dying process is as described here. The cell culture media was removed. The cell tracker working solution was added in a volume which covers the total surface area of the cell culture. The cell culture was incubated with the working solution for 30 minutes incubation with Cell Tracker Green and 15 minute incubation with Cell Tracker Red. The cell tracker was removed and replaced with normal cell media. These cells are now the '**dyed cells**' and need incubation for at least 30 minute for use. Once this staining process was completed, the cytoplasm of the dyed cells may be used at will.

Basic Methods – Summarized steps

1. Warm media in the water bath
2. Use a needle tip and syringe to pull out 1 mL of DMSO and placed in a 15mL conical
3. Use micropipette to pull out 10.8µL of DMSO from the 15mL conical
4. Add the DMSO to the vial of Cell Tracker – attempt to get most of the powder cell tracker dye in with the DMSO. This is a 10mM concentration of **stock solution**, via the following equations:

$$M = \frac{\text{moles}}{L} \quad \text{mole} = \frac{g}{w} = \frac{.05g}{464.86} = 1.08 \times 10^{-4} \text{ moles}$$

$$0.01M = \frac{1.08 \times 10^{-4} \text{ moles}}{L} \quad L = 1.08 \times 10^{-2} L = 10.8 \text{ mL}$$

5. Dilute to the desired concentration for the cell tracker dye to create the **working solution**
 - Use the equation $c_1 v_1 = c_2 v_2$ (where $c_1 = 10\text{mM}$ – from the stock solution, $v_1 =$ is unknown, $c_2 =$ the desired concentration – in this case $5\mu\text{M}$, and $v_2 =$ the final volume needed)
$$10\text{mM}(x) = 5\mu\text{M}(19\text{mL})$$
 - a. $10000\mu\text{M}(x) = 5\mu\text{M}(19\text{mL})$

$$x = .0095\text{mL} = 9.5\mu\text{L of Stock solution}$$
6. Vortex the working solution for use
 - a. Solution can be stored in the fridge for up to one week

7. Remove the media from the cell culture
8. Add your working solution of the Cell Tracker Dye to the cell culture
 - a. Cell Tracker Green should be incubated on the cells for 30 min
 - b. Cell Tracker Red should be incubated on the cells for 15 min
9. Remove the Cell Tracker Dye working solution from the cell culture
10. Add cell media back onto the cell culture
11. Incubate for at least 30 minutes
12. Cells are now dyed ('**dyed cells**') and able to be used as you wish – i.e. pressure sodding described below
 - a. The dyed cells can be used for about 1 week (different concentrations will produce different intensities, as characterized by the graph below) as the dyed cells proliferate either through passages or sodding.

The Pressure Sodding

The checklist protocol for this experiment can be seen in Appendix 1. Human Umbilical Vein Endothelial Cells (hUVECs) were cultured to 90% confluency. Cells were stained with a 15 μ M concentration of Cell Tracker Green following the *basic methods*. The cells were trypsinized and sodded onto two grafts. The grafts were exposed to flow and pressure sodded at 8 million cells per vessel (vessel length, 3 cm). After sodding, vessels were placed on a peristaltic pump with transmural flow at 10 rpm for 1 hour, no rotation. Then, flow was averted lumenally at 10 rpm for 24 hours. The vessels were taken down by cutting them from the barbs using a blade. In this experiment no fixation occurred, images were taken right after the vessels were taken down. It is unsure how histochoice will effect the concentrations of the dye, this will need to be investigated in later experiments. To image the vessels a longitudinal cut was made creating semi circular halves. The endothelium was exposed for imaging from proximal to distal, as seen in Figure 1. As a clear view of the cellular lining is in focus on the microscope, take an image for both the green and red filter wheels for each portion of the vessel. Cell Tracker Green is imaged using Turret-4, S1, FW1-3, and FW2-2 and Cell Tracker Red is imaged using Turret-4, S1, FW1-4, FW2-3. Vessels were imaged on an Olympus BX41 microscope with motor x-y stage for wide field fluorescence.

Appendix C: Gene Expression Protocols

ePTFE Vessel Take-Down Protocol

Before removing bioreactor from incubator prepare the following:

- ___ 37°C PBS in a trough
- ___ 10mL syringe
- ___ Razor blade
- ___ Liquid Nitrogen in small dewar
- ___ 15mL conical (1 per vessel)

Take-Down:

- ___ Close all valves
- ___ Remove reservoir and tubing from chamber
- ___ Remove lid from chamber and drain bioreactor media, DO NOT TOUCH VESSEL!
- ___ Slowly flush transluminally with 2-4mL of PBS
- ___ Slowly flush transmurally with 6-8mL of PBS, or until vessel is visibly clear of all media
- ___ Cut out vessel and fix in 15mL conical in liquid nitrogen

NOTES:

- Fix vessels immediately, DO NOT cut scaffolds longitudinally until after fixation
- Transfer vessels to -80°C as soon as possible after complete freezing

RNA Isolation protocol – Use RNeasy Mini Kit and Instructions from Quiagen

1. Rinse/Fix scaffolds in liquid nitrogen according to protocols for the given material
2. In RNase-free tube:
 - a. Insert scaffold, cut to size
 - b. Add 600µL of Buffer RLT (cell lysate)
 - c. Agitate/vortex for 5 min
3. Transfer lysate (fluid) directly to a QIAshredder spin column for homogenization
4. Spin QIAshredder tube at 161000rcf (16.1k) for 2 min
5. Add 600µL of 70% ethanol to the lysate
6. Pipette to mix, add 600µL to RNeasy spin column
7. Spin for 15sec at 16.1k rcf, discard flow-through
8. Repeat 6,7 for remaining lysate
9. Add 700µL of RW1 to the spin column
10. Spin for 15sec at 16.1k rcf, discard flow-through
11. Add 500µL of Buffer RPE to spin column
12. Spin for 15sec at 16.1k rcf, discard flow-through
13. Add another 500µL of Buffer RPE to spin column
14. Spin for 2min at 16.1k rcf, discard flow-through
15. Spin column empty for 1min (drying step)
16. Place RNeasy spin column in new 1.5mL collection tube
17. Add 30µL of RNase-free water directly to the membrane
18. Spin for 1min at 16.1k rcf
19. Pipette the flow-through back onto the membrane (to concentrate the RNA)
20. Spin for 1min at 16.1k rcf
21. Use NanoDrop Spectrophotometer to quantify concentration and get information of RNA quality/purity (see NanoDrop Spectrophotometer protocol)

NanoDrop Spectrophotometer Protocol

1. Turn on the computer, select the NanoDrop icon on the desktop
2. Select Nucleic Acid as the sample type
3. On the User Interface, select RNA as the sample type (DNA is the default)

USING RNase FREE WATER AND KIMWIPES:

4. Use 1uL of water to clean/wipe the stage
5. Apply 1uL to the stage, lower the arm, and blank the spectrophotometer
6. Dry the stage
7. Name the sample (Initials, sample ID, date)
8. Flick to mix the RNA sample
9. Apply 1uL of sample to the stage
10. Hit play
11. Record results (Concentration, 260/280 and 260/230)
12. Dry the stage
13. Apply 1uL of water to the stage, lower the arm
14. Raise the arm, dry both top and bottom of stage
15. Repeat steps 7 through 13 for all samples

Primer Design

1. Go to GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>)
2. Select Nucleotide from the drop down menu
3. Search for the gene of interest, ensuring that the human protein (Homo sapiens) is selected
4. Ensure that mRNA is selected
5. Scroll to the bottom of the page to locate the sequence
6. Select the last half to third of the sequence (depending on the overall length of the gene)
7. Go to Primer3 (<http://frodo.wi.mit.edu/>)
8. Copy the sequence into the box at the top of the page (it will automatically ignore the line numbers)
9. Settings for Primer3:
 - a. Pick left and right primers
 - b. Set "Product Size Range" to 180-220
 - c. Set "Primer Size" Min to 18, Max to 22
 - d. Set "Primer Tm" Min to 59.0, Max to 61.0
 - e. Set "Primer GC%" Min to 30, Max to 80
10. Click "Pick Primers" button
11. Go to Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>)
12. Enter forward and reverse primers into Primer Parameters box
13. Check for homology with non-target genes, if found select different primer set from Primer3 and repeat
14. In general:
 - a. Want high homology with different variants of same gene, to ensure that transcript variation does not affect results
 - b. Want low homology with non-target genes, if primer has less than four mismatches with a non-target gene, pick new primers
15. Go to Integrated DNA Technologies (<http://www.idtdna.com/site>)
16. Select Products → DNA/RNA Synthesis → Custom DNA Oligos
17. Select Multiple Entry, enter the number of primers required
18. Change Normalization to "LabReady"
19. Change Scale to 25nmol DNA oligo
20. Change Purification to Standard Desalting
21. Name Sequence with gene and forward or reverse
22. Note the size of the expected amplicon
23. Place order, each primer pair should be approximately \$20 (\$10 each forward and reverse)

Reverse Transcription.

- Find the amount of RNA sample needed in each tube by using the following equation to get a final 2000ng of RNA per tube.
 - $2000\text{ng} / [\text{RNA sample in ng / uL}] = V_{\text{sample}} \text{ uL of sample}$
 - Ex. Given a [] of 926ng/uL $V_{\text{sample}} = 2.16\text{uL}$
- Determine how many tubes can be made with a given RNA [].
 - $V_{\text{total}} (\text{of RNA sample} \approx 25\text{uL}) / V_{\text{sample}} = \# \text{ tubes.}$ *be sure to leave room for 10% extra*
- Find the total amount of sample, water, accuscript, gene specific primer, and dNTP needed per tube.
 - Add 2uL Accuscript for each tube.
 - Add 1uL gene specific primer to each tube.
 - Add .8 uL dNTP for each tube.
 - Determine amount of water to total 12.8 uL (for water + sample).
 - $12.8 - V_{\text{sample}} = V_{\text{water}}$
 - Ex. If $V_{\text{sample}} = 2.16\text{uL}$ the $V_{\text{water}} = 10.64\text{uL}$
- To begin preparing master mixes, multiply amounts of each component by # tubes to be made, then again by 1.1 to add 10% for error.
 - Ex. 2uL Accuscript for 10 tubes becomes $2\text{uL} \times 10 \times 1.1 = 22 \text{ uL}$
- Begin adding all the components of the master mix together.
 - First add water (because it is the largest volume)
 - Next add buffer, primer, and dNTP (vortex primer and dNTP before use)
 - Last add template (flick to mix).
- Alloquat 16.5 uL of each master mix into each tube.
- Put tubes in thermal cycler (avoiding edges). Edit the program to be:
 - 65°C for 5 mins
 - 20°C for 3 mins
 - sample vol = 17uL.
- While thermal cycler is running, prepare next master mix of (multiply amounts of a, b, and c by # tubes +1)
 - 2uL DTT
 - 1uL AccuScript
 - .5uL RNase block
- When thermal cycle is done add 3.5uL of master mix to each tube. Spin down each tube briefly ~5sec.
- Reload thermal cycler and run a Program as follows:
 - 42°C 60min
 - 72°C 15 min
 - 4°C forever.
 - Total sample vol = 20uL.
- Store cDNA at -20°C.

QPCR protocol:

*Take all necessary precautions to keep plate and components of reaction cold throughout preparation.

1. Prepare a master mix for each primer of each sample. For each sample, you will need a minimum of 3 replicates of each primer. A master mix for 3 reactions consists of the following:
 - a. 41.25uL SybrMM
 - b. 2.475 10uM primer
 - c. 13.2 cDNA template
 - d. 25.575 water

*this recipe allows 25 uL per well plus 10% error
2. If you are preparing a reaction for one well then the mix consists of:
 - a. 12.5uL SybrMM
 - b. .75uL of 10uM primer
 - c. 7.75uL water
 - d. 4uL template * for a nontemplate control use these amount but replace the 4uL template with 4uL water.
3. Prior to prepping your plate design the Thermal Cycler Program on laptop to be:

Stage	Rep	Temp	Time	Ramp
1	1	95.0°C	15:00	Auto
2	40	94.0°C	:15	
		58.0°C	:30	
		72.0°C	:30	

Then click the add dissociation step. And change the sample size to 25uL from 20uL.

4. Prepare master mixes, adding the Sybr last.
5. For each primer prepare one reaction of NTC.
6. Pipette 25uL of each master mix into a well, keeping track of where each primer/sample is located as well as the NTC.

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