***Tissue Engineering A Blood Vessel Mimic While
Monitoring Contamination Through Sterility Assurance Testing***

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By

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Abstract

Tissue Engineering A Blood Vessel Mimic While Monitoring Contamination Through Sterility Assurance Testing

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Tissue engineering blood vessel mimics has been proposed as a method to analyze the endothelial cell response to intravascular devices that are used in today's clinical settings for the treatment of cardiovascular disease. Thus, the development of *in vitro* blood vessel mimics (BVMs) in Cal Poly's Tissue Engineering Lab has introduced the possibility of assessing the characteristics of cellular response to past, present, and future intravascular devices that aim at treating coronary artery disease.

This thesis aimed at improving the methods and procedures utilized in the BVM model. Initial aspects of this project focused on using an expanded polytetrafluoroethylene (ePTFE) scaffold in conjunction with human endothelial cells to replicate the innermost intimal layer of a blood vessel. Human umbilical vein endothelial cells (HUVECs) were pressure seeded onto ePTFE scaffolds through cell seeding techniques in an attempt to effectively and consistently replicate and assess the intimal layer. Through each study ePTFE grafts were subjected to different culture times and steady flow rates to observe and compare the differences in the endothelial cell deposition. Results were inconsistent, although moderate cell adhesion was noted

throughout each of the BVM setups. Each study exhibited a range of cell sodding density rates.

In the second phase of the thesis, contamination assessment protocols were implemented in the BVM lab. The intent of this part of the project was to assess the relative sterility in the cell culture lab, a critical component involved with the success or hindrance of cell and tissue cultures. Using microbiological validated methods, microbiological tests were conducted to examine the levels of microbial growth in and around the tissue engineering lab.

Results were tracked over a two month period in the lab with several observations of aerobic microorganism growth on various counter and lab surfaces. Higher growth trends were found on surfaces outside the cell culture lab, in the general TE lab area. These findings were used to provide overall suggestions on tracking microbes for long-term durations in ongoing BVM setups to directly improve the overall sterility assurance of the studies.

As the project reached its conclusion a look back at all the BVM setups and contamination assessments lead to a few suggestions for improving aseptic techniques within the TE lab, such as monitoring microbial growth in the culture processes, creating limit specifications, and creating a standardized way to regulate quality control within the lab environment. Furthermore, as the development BVM evolves, the findings from this report can be used with related research for improving the culture conditions of various BVM studies.

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Table of Contents

List of Figures	xiii
List of Tables	xvi
CHAPTER 1: Introduction	1
1.1 Overview	1
1.1.1 <i>Blood Vessel Biology and Atherosclerosis</i>	3
1.1.2 <i>Treatment Therapies and Justification of TEVGS</i>	4
1.1.3 <i>Tissue Engineering Impact</i>	7
1.2 The Aims and Methodology of Tissue Engineered Vascular Grafts	7
1.2.1 <i>Introduction</i>	7
1.2.2 <i>Relevant Experimental Fields</i>	7
1.2.3 <i>BVM Research</i>	8
1.2.4 <i>Graft Compliance and Biocompatibility</i>	9
1.2.5 <i>Scaffold Development</i>	12
1.2.5.1 <i>Scaffold Development with Well-Plate and Electrospun Scaffolds in Static Culture Conditions</i>	12
1.2.5.2 <i>Dynamic Shear Flow Environments</i>	16
1.2.6 <i>Vessel Conditioning</i>	18
1.3 Isolation and Cultivation Techniques of HUVECs	20
1.3.1 <i>Isolation Methods</i>	20
1.3.2 <i>Characterizing Viable Culture and Growth Techniques</i>	23

1.3.2.1	<i>Application of Different Growth Media Conditioning</i>	24
1.3.2.2	<i>HUVEC Cultures</i>	28
1.4	HUVEC Development in TEVGs	29
1.4.1	<i>In Vivo Studies</i>	29
1.4.2	<i>HUVEC Development and Response in Static and Shear Environments</i>	31
1.4.3	<i>HUVEC Development on Stent Materials</i>	35
1.4.4	<i>Effects of Scaffold Surface Roughness</i>	37
1.4.5	<i>Growth Factor Influence in HUVEC Cultures</i>	40
1.5	Methods for Cell Delivery onto Scaffolds and TEVGs	41
1.5.1	<i>Cell Sodding/Seeding Techniques</i>	42
1.5.2	<i>HUVEC Sodding Trials in ePTFE Scaffolds</i>	44
1.6	Summary and Aims of the Thesis	45
Chapter 2: Blood Vessel Mimic (BVM) Set-Ups Over One, Three, and Seven Days		48
2.1	Introduction to Blood Vessel Mimics	48
2.1.1	<i>History of Blood Vessel Mimics</i>	48
2.2	Materials and Methods	49
2.2.1	<i>Materials and Methods for Study One</i>	49
2.2.2	<i>Materials and Methods for Study Two</i>	55
2.2.3	<i>Materials and Methods for Study Three</i>	56
2.3	Results and Observations	56

2.3.1	<i>Results from Study One</i>	56
2.3.1.1	<i>BBI Imaging</i>	58
2.3.1.2	<i>Histology</i>	61
2.3.2	<i>Results from Study Two</i>	65
2.3.2.1	<i>BBI Imaging</i>	66
2.3.2.2	<i>Histology</i>	69
2.3.3	<i>Results from Study Three</i>	74
2.3.3.1	<i>BBI Imaging</i>	75
2.3.3.2	<i>Histology</i>	81
2.4	Conclusion of BVM Studies	89
2.4.1	<i>General Observations of BVM Studies</i>	89
2.4.2	<i>Study One Discussion</i>	90
2.4.3	<i>Study Two Discussion</i>	91
2.4.4	<i>Study Three Discussion</i>	93
2.4.5	<i>Future Outlook/Next Steps</i>	94
Chapter 3: Sterility Assurance of BVM Systems through		
Microbiological Practices		96
3.1 Introduction to Microbial Testing Significance and		
Microbiological Assessments		96
3.1.1	<i>Early Forms of Microbiological Testing and Bacterium Growth</i>	96
3.1.2	<i>Testing Bacteria Presence in Modern Era</i>	97
3.1.3	<i>Endothelial Cell Responses to Bacteria Exposures and Contamination</i>	98

3.2 Cell Culture Contamination: Identification and Testing

Methods _____ 99

3.2.1	Contamination Sources	99
3.2.1.1	Biological Sources	100
3.2.1.1.1	Mycoplasmas	101
3.2.1.1.2	Mycoplasma Contamination Testing with PCR methodology	101
3.2.1.2	Aerobic Microorganisms (MOs) and Coliform Groups	102
3.2.1.3	Staphylococcus Aureus (S. Aureus)	103
3.2.2	Chemical and Physical Sources	104
3.2.3	Consequences and Effects	106
3.2.4	Control, Prevention, and Sterile Barrier Systems	106
3.2.5	Assurance Techniques and Limits	108

3.3 Testing Implementation _____ 110

3.3.1	ISO Testing Methods	111
3.3.2	AOAC, and HACCP Testing	112

3.4 Materials-Methods _____ 113

3.4.1	Overview	113
3.4.1.1	Swab Contact Method	115
3.4.1.2	Sponge Contact Method	115
3.4.1.3	Aerobic Plate Count (APC)	115
3.4.1.4	Coliform Count, E.Coli, and Staph Plate Count	116
3.4.2	Cleanliness Testing of Researchers Hands & Gloves	116
3.4.3	Sterility Testing of Glassware, Equipment, and Media	117

3.5 Results - Tracking _____ 117

3.5.1	<i>Interpretation</i>	117
3.5.2	<i>APC Analyses, EC/TC Counts, Staph Counts</i>	118
3.6	<i>Discussion</i>	120
Chapter 4:	<i>Discussion/Conclusion</i>	123
4.1	Discussion of BVM Studies	123
4.2	Progression of BVMs studies and Bioreactor Culture Systems	125
4.3	Discussion of Sterility Assurance Monitoring with BVM Utilization	126
4.4	Future Goals	129
<i>Bibliography</i>		130
<i>Appendices</i>		138
Appendix A - Study One BVM Setup - Fall '09: December, 2009		138
Appendix B - Study Two BVM Setup - Summer '10: June, 2010		150
Appendix C - Study Three BVM Setup - Summer '10: Sept. 1st, 2010		161
Appendix D - Histology Protocol: Study One and Study Two		171

Appendix E - Histology Protocol Study Three	173
Appendix F - HUVEC Cell Average Counts (Study Two and Three)	175
Appendix G - BBI (Bisbenzimidazole Imaging) Protocol	183
Appendix H - Microbiological Testing Protocols	184
Appendix I - <i>Media recipes</i>	210
Appendix J - Microtome Protocol	211
Appendix K - HUVEC Density Values for BVM Studies	212

List of Figures

Figure 1: Diagram of the 3 major layers of a blood vessel	4
Figure 2: A coronary artery with plaque buildup	6
Figure 3: Radiograph of a fully deployed stent inside a cultured BVM vessel	10
Figure 4: SEM images of HCASMCs under static and dynamic condition	19
Figure 5: SEM images of sixth passage HUVECs	27
Figure 6: TUD-1 and serum-containing media in HUVEC cultivation	28
Figure 7: Morphological images of HUVEC-seeded graft	35
Figure 8: A growth index of HUVECs on metallic materials	37
Figure 9: HUVECs on polymer modified materials	40
Figure 10: A dual-loop bioreactor used in TEVG cultures	51
Figure 11: A HC34 Taylor-Wharton N ₂ Tank	51
Figure 12: Shel Lab incubator operating at 37°C and 5% CO ₂	51
Figure 13: CKX31 Optical Microscope	52
Figure 14: Media reservoir assembly	52
Figure 15: Bioreactor chamber with accompanying tubing	52
Figure 16: M9 Ultraclave Autoclave	53
Figure 17: Autoclave with media flask	53
Figure 18: View of the bioreactor setup	54
Figure 19: Multiple views of the completed BVM setup	55
Figure 20: Bioreactor system configured with respective media reservoir	55
Figure 21: Proximal-middle region of vessel A	59
Figure 22: Middle-distal region of vessel B	60
Figure 23: Vessel A at the distal region	60
Figure 24: Vessel C cultured at 72 hours	60
Figure 25: Vessel D cultured at 72 hours	61
Figure 26: Middle-proximal image Vessel E	61
Figure 27: Middle-distal shot of vessel D	62
Figure 28: Vessel E at the distal end	62
Figure 29: Vessels A at 4x magnification	63
Figure 30: Vessel B with cell seeding density of 2.5×10^6 cells/cm ²	63
Figure 31: Left proximal section of Vessel A at 10x	63
Figure 32: Right proximal view of Vessel B at 10x	63
Figure 33: Left angle proximal view of vessel C	63
Figure 34: Vessel C at 4x magnification	63
Figure 35: Vessel C from the center vessel cross-section	64
Figure 36: Vessel C at 10x	64
Figure 37: Vessel E cultured for 1 week	64
Figure 38: Top proximal view of Vessel E	64
Figure 39: HUVEC adhesion along the ePTFE intersitices of Vessel F	65
Figure 40: Cross sectional view of Vessel F	65
Figure 41: Vessel F at 4x	65

Figure 42: Right centered proximal view of Vessel E	65
Figure 43: Vessel F at 10x magnification	66
Figure 44: Vessel E cultured at 10x	66
Figure 45: Proximal regions from vessel 1	67
Figure 46: Original middle region of the BVM vessel 1	68
Figure 47: Middle-proximal view of vessel 1	68
Figure 48: BVM image of the right-distal region	68
Figure 49: Vessel near proximal-mid region	68
Figure 50: BVM at mid-distal range	69
Figure 51: An outer edge BBI shot of the vessel 1	69
Figure 52: Vessel 1 mid-distal region	70
Figure 53: The 24-hr BVM at 4x magnification under a white light	71
Figure 54: A bottom middle-proximal view of vessel 1	72
Figure 55: Cross sectional area of the BVM	72
Figure 56: BVM histology presented at a 10x views	73
Figure 57: Colony grouping within the ePTFE vessel	73
Figure 58: Development of an endothelial lining	73
Figure 59: Right region of the vessel walls	73
Figure 60: A zoomed view of the middle-top area of the cross section	74
Figure 61: Stacked effect of the cultured HUVECs on the scaffold lumen	74
Figure 62: Adjusted view of the endothelial lining	74
Figure 63: Shows vessel 1 at the distal end of inner wall with also minimal cell coverage	76
Figure 64: Vessel 1 at the proximal-mid region	77
Figure 65: Proximal-distal area of vessel 1	77
Figure 66: A mid-distal shot of vessel 1	77
Figure 67: Mid-distal area of vessel 2	77
Figure 68: Proximal area of vessel 2 fragment.	77
Figure 69: Distal regions of vessel 3	78
Figure 70: Middle region of vessel 3	78
Figure 71: Proximal-mid region of vessel 3	78
Figure 72: Vessel 3 further along the mid region	78
Figure 73: Expanded view of the prox-mid region of vessel 3	79
Figure 74: HUVEC presence near the proximal region	80
Figure 75: BBI image of vessel 4	80
Figure 76: Proximal end of vessel 4	80
Figure 77: Prox-middle region with endothelial development	80
Figure 78: Middle view of the prox-mid area of the vessel 4	81
Figure 79: Bottom area of the mid-distal region of vessel 4	81
Figure 80: Average cell count of the proximal-mid area of the BVM vessel 4	82
Figure 81: Cross sections of vessel 1	83
Figure 82: Vessel 1 histology	83
Figure 83: Vessel 1 at 10x view	84

Figure 84: Right mid-proximal side of vessel 1	84
Figure 85: A right mid-proximal view of vessel 2 at 4x magnification	84
Figure 86: Top-mid shot of the BVM vessel 2	84
Figure 87: Vessel 2 cell lining development	85
Figure 88: Evidence of cell adhesion on vessel 2	85
Figure 89: HUVEC adhesion on vessel 2	85
Figure 90: Vessel 2 cross sections cultured at 10x	85
Figure 91: A 10x image of Vessel 2	86
Figure 92: HUVEC grouping along vessel 3	86
Figure 93: Circumferential snapshots of vessel 3	86
Figure 94: Vessel 3 at 4x magnification	87
Figure 95: Moderately developed cell lining across vessel 3	87
Figure 96: Mid-proximal regions of vessel 3	87
Figure 97: Consistent cell lining along and middle top luminal area of vessel 3	87
Figure 98: HUVEC lining along vessel 3 cross section	88
Figure 99: Endothelial development of vessel 3	88
Figure 100: Vessel 4 cell lining networks along the lumen wall	88
Figure 101: Vessel 4 presented from top-mid view	88
Figure 102: Vessel 4 presented from the bottom-mid region	89
Figure 103: Middle-low angle view the endothelial of vessel 4	89
Figure 104: A 10x view of the bottom rim of vessel 4	89
Figure 105: Cell stacking along vessel 4	89
Figure 106: Vessel 4 images just above the folded region of the graft cross section	90
Figure 107: A closer view of the left middle-proximal region of vessel 4	90
Figure 108: HAACP Testing Flowchart	113
Figure 109: EC/TC petrifilm count	119
Figure 110: APC petrifilm Count	119

List of Tables

Table 1: Key features of a small diameter blood vessel	12
Table 2: Study One cell confluency and cell sodding density values	58
Table 3: Study Two BVM culture parameters	67
Table 4: Study Three BVM culture parameters	75
Table 5: Microbial counts of cell culture and general laboratory	119
Table 6: Microbial counts of cell culture and general laboratory	120
Table 7: List of Sterilized Materials	153
Table 8: Media Quantities used in Study Two and Study Three	153

CHAPTER 1: Introduction

1.1 Overview

The demand for blood vessel substitutes is rapidly increasing as patients require the need to replace coronary and peripheral vessels due to circulatory diseases such as atherosclerosis. The transition from using autologous and synthetic vessels to biologically engineered vessels is presently more foreseeable [1]. Tissue engineered vascular graft (TEVG) researchers have closely studied the intricacies of blood vessel fabrication in the past few years leading the way to the development of possible blood vessel substitutes in the laboratory. Using tissue engineering (TE) cell culture protocols developed in recent years, progress has been made in understanding how blood vessel components work together and respond when cultured in tubular-shaped polymer scaffolds placed in various controlled environments [2]. However, the conclusions from past successful trials in tissue engineering experiments have yet to come together to consistently produce a fully functioning and clinically applicable tissue engineered blood vessel. Recreating one native feature at a time is the first step of many to reach a highly valuable replicate biologic vessel.

The overall goal of this thesis project was to improve current blood vessel mimic (BVM) methods developed in the Cal Poly TE lab through experimental trials looking to create the most consistent endothelium and introducing sterility assessment techniques. After initial background research, the focus shifted towards building a consistent BVM through a proven working protocol. To accomplish this, the first aim was to explore the

techniques for producing an *in vitro* replica of an endothelial using human umbilical vein endothelial cells (HUVECs). A proposed confluent EC layer inside an expanded-polytetrafluoroethylene (ePTFE) scaffold served to mimic the intima layer of a blood vessel. This part of the project tracked specific changes in the BVM protocol to provide ideas on areas for possible protocol improvement.

The second aim of this project was to initiate a contamination risk assessment of the TE lab, which was identified as one possible area for improvement within BVM cultivation. Microbial contamination is a significant inhibitor of cell culture practices and thus a major key to the success of TEVG studies. Therefore the testing practices introduced served to improve the concept of maintaining a controlled sterile working environment. Microbiological approved USDA and AOAC methods were implemented for an extended period in the TE lab to compare and track results to assess the sterility, and overall cleanliness of the TE lab.

To provide background on the aims of the project, as well as TEVGs and how they have contributed to the development of BVMs in the TE laboratory, several areas of interest will be covered in the introduction. First, a review of blood vessel biology will be covered leading up to the discussion of TEVG methodology. Then several topics are presented in hopes of linking key features of TEVGs and the importance of the experimental parameters that will be summarized in the methods portion of the BVM studies. Finally, at the end of this chapter, an overall summary will be provided along with the specific goals of the thesis project.

1.1.1 Blood Vessel Biology and Atherosclerosis

There are three primary types of blood vessels: arteries that carry oxygenated blood away from the heart, capillaries that allow the transport of water and other nutrients between blood and tissue, and veins that carry deoxygenated blood from capillaries back to the heart. Figure 1 shows the composition of a native blood vessel, which consists of three main layers; the adventitia, media, and intima [3]. As the outermost blood vessel layer, the adventitia consists mainly of connective tissue, vasa vasorum, and nerves that supply the muscular layer. The middle layer called the media, consists of smooth muscle cells (SMCs), and elastin. Making up the innermost intima layer is the endothelium, a thin layer of single squamous endothelial cells (ECs), and a basement membrane. The endothelial layer directly contacts shear stresses due to blood flow across the luminal surface. Also, the endothelium is thought to provide tissue homeostasis, regulation of vascular tone, and inhibit thrombosis potential [1]. What makes the endothelial layer relevant to cardiovascular therapeutics such as stents, are the critical EC responses that occur throughout the lumen region with an implanted device. Thus, being able to fully understand the behavior of an endothelial lining and its characteristics in different environments is crucial to the future success of BVMs, and evaluations of applicable medical devices in a preclinical setting.

Figure 1: Diagram of the 3 major layers of a blood vessel: the outer adventitia layer, the middle media layer, and the inner intima which consists of an endothelium wrapped in an elastic internal lamina [3].

1.1.2 Treatment Therapies and Justification of TEVGS

Currently, a large focus in the medical field is concerned with the number one killer in the United States; cardiovascular disease [1]. A specific cardiovascular disease that is rising in the Western world is atherosclerosis [1, 2]. As blood flows away from the heart into arteries, it may experience resistance due to the buildup of plaque or fatty material. When plaque accumulates along the arterial wall it can harden and eventually block the artery, leading to a potential myocardial infarction, which is commonly known as a heart attack.

Improving the assessment of treatment therapies in cardiovascular disease is necessary to create a highly efficient and effective output of current cardiovascular medical devices therapies [4]. Tissue engineering cardiovascular tissue is just one example that can provide researchers with valuable information about how clinical treatments work in an *in vitro* setting prior to implantation. This approach could eliminate a lot of medical research cost and reduce the amount of animal testing that is needed to justify the use of intravascular medical devices, such as the response of drug coated stents on human arteries [4]. Before an accurate evaluation can be made, small steps must be taken to replicate the dynamic biological actions and cellular components of living blood vessels; a hurdle that begins with the successful creation of an endothelial layer inside a tissue engineered BVM.

Ultimately, the scientific community is searching for reliable engineered replacement tissues for coronary or peripheral bypass procedures. Until that day, current stent technologies provide patients of atherosclerosis the best possible alternative to invasive surgeries such as coronary bypass [2]. The figure below describes basic stent technology by using a deployed balloon catheter inside an occluded area of a coronary artery. Once the catheter is removed, the stent is fixed in the artery and pushes the plaque buildup against the walls allowing blood flow to resume in that region of the artery [5]. This treatment procedure is not always a permanent solution to a clogged artery, but provides at least temporary relief to the patient, which could last a lifetime in ideal cases. Problems that arise in stent

placements can vary from restenosis or reoccurrence of vessel narrowing, thrombosis near the stent position, and re-vascularization due to stent failure.

Figure 2: The first stages of the diagram show a coronary artery with plaque buildup. Later, a stent mounted on a balloon is guided with a catheter and is inflated as illustrated. Once inflated, the plaque is pushed to the side and blood flow through the area is restored [5].

Understanding the problems of cardiovascular treatment therapies can be addressed from continued breakthroughs by TEVG research and related engineered blood vessel models. Further investigations into various problems that develop inside

blood vessel substitutes will assist the development of TEVG cultures. BVM modeling looks to develop methods for accurate vessel layer constructs and further assessment with stent-device interaction [4, 6]

1.1.3 Tissue Engineering Impact

As scientific research advances, tissue engineering is forecasted as a field with limitless potential in regenerative medical therapy as seen specifically with current trials to develop optimal tissue grafts for clinical evaluation purposes [7, 8]. As the world of science and medicine becomes more complex, future research will play an increased role in the development of viable alternatives to pharmacological resources and assistive medical devices in repairing biological dysfunctions: inherited and non-inherited.

Tissue engineering pulls knowledge from multidisciplinary cell biology and engineering sciences to provide a potential solution for incurable diseases, failing organ systems, and age-related health problems. Cardiovascular disease such as atherosclerosis is one example of many age-related health problems that continues to gain understanding through current TEVG experimental studies. The following TEVG studies focused on various aspects of the culture process that were critical to the development of incorporated cell lines as well following up on studies with respect to previous TE trials in hopes of gaining vital information on the recreation of TEVG components.

1.2 The Aims and Methodology of Tissue Engineered Vascular Grafts

1.2.1 Introduction

Tissue engineered vascular graft (TEVG) research is crucial to successful vessel creation because it allows researchers to review what experimental conditions within a protocol affected the development of vessel components in a particular environment. Some current fields that are of interest at this point in TEVG research include: cell signaling mechanisms due to mechanical stimuli, and adaptability in different culture systems [2, 8]. However, a refined TEVG process can expand the blueprint for current researchers to reference, for example when examining the key factors required in producing a functioning endothelium; in this case within blood vessel constructs.

1.2.2 Relevant Experimental Fields

Exploring the complexity of TEVGs, a further look into specific parameters that are utilized in replicating blood vessels and related tissue constructs will be a prelude to the sections examining BVM methodology. Moving forward, these related fields will supplement discussions for pertinent questions that surround the BVM model, and how endothelial formation can be improved within the vessel constructs created.

After discussing a few intricacies of BVM modeling, important topics such as scaffold preparation, EC viability, endothelial layer development, and the types of cell culture systems will be reviewed. These sections will summarize topics through TEVG

case studies; beginning with a recent BVM model and introducing the idea of graft compliance. Later, cell seeding techniques and TEVG construction with EC cultures will be explored.

1.2.3 BVM Research

In recent years, there have been advancements in TEVGs as a unique preclinical assessment of how a stent will interact with a blood vessel in an *in vitro* setting [4, 6, 9]. The endothelial layer is recreated, turning it into an *in vitro* “blood vessel mimic” (BVM) to evaluate stent endothelialization within a blood vessel. Figure 3 shows a radiograph image of a deployed stent that was studied in a BVM that was cultured in a small-scale bioreactor system under steady peristaltic flow [4]. Scanning electron microscopy (SEM) verified a cellular lining and the presence of a luminal monolayer of ECs inside the BVM [4]. Such preclinical breakthroughs have helped the improvement of medical assessments of vascular cells with 21st century stents such as drug-eluting stents, protein coated stents, and stent grafts inside mimicked blood vessels. However, before vital stent assessments inside a BVM can be universally accepted, the most appropriate biologic vessel must be recreated and reformed to give the best possible data regarding such therapeutic technologies.

Further insight into BVM related research will be revisited in the following chapter as well as introductions to cell culture practices that have been employed in similar TEVG methods. Leading up to studies of relevance; the topics discussed will relate concepts incorporated in BVM research that will collectively contribute to TEVG studies.

Figure 3: A radiograph is used to help assess the stability and structure of a fully deployed stent inside the cultured BVM vessel [4].

1.2.4 Graft Compliance and Biocompatibility

Expanding on the idea of recreating the best possible TE blood vessel, the importance of a vessel's compliance and mechanical properties can contribute in the degree of model accuracy of future stent evaluations. An ideal scaffold should mimic the ability of a vessel to function properly within systems that have native co-cultured blood vessel cells [1]. Compliance mimicking from synthetic to native vessel properties revolves around understanding the mechanical system with which vessels respond to in dynamic blood flow, or a person's physical body movement, and the adjustment of a blood vessel over time and stress.

Generally, researchers have investigated what parameters influence the mechanical integrity of a TEVG over different culture periods. Compliance mismatch of replacement vessels is correlated to the induction of intimal hyperplasia which is in part due to the limited elastin production in tissue engineering approaches [10, 11]. Furthermore, an optimally designed replacement TEVG would be able to mimic the

native vessel's compliance by recoiling appropriately after feeling compressive and distending pressure forces [11].

The degree of compliance of a tubular graft relies heavily on the innate mechanical properties of the scaffold and the changes of those properties through its intended life time [2]. With the incorporation of biological components into TEVGs, there have also been synthetic and natural polymers used to represent the native vessel. While compliance features such as burst strength and hyperplasia of cells have not been measured with the current BVM model, introducing compliance values during scaffold fabrication may enhance the culture environment by incorporating a scaffold that is more physiologically accurate and responsive to dynamic testing environments with respect to a native vessel.

Expanding on compliance mimicking, the vasoactivity of native TEVGs continues to be a challenge to recreate in blood vessel substitutes. With native blood vessels, ECs receive and send neuronal signals that modulate vascular tone, while smooth muscle cells (SMCs) generate the contractile force necessary to change vessel volume and blood pressure by responding to nervous and hormonal signals [2]. SMCs tend to undergo a transition to a synthetic phenotype, in which the cells lose their contractile function, when they have been cultured within *in vitro* constructs [12, 13]. A clearly defined set of compliance values can aid the endeavors of recreating these characteristics. Most approximate values come from comparable values of specific biologic blood vessels that are modeled or tested. Table 1 lists some characteristics of

an ideal small diameter blood vessel (SDBV) replacement including descriptions on compliance matching.

Table 1: A review of key physical compatibility and processing features that a SDBV or generalized TEVG can contain. The most critical characteristics are listed near the top and trend downward [7].

Physiological
Withstand at least 80–120 mm Hg physiological pressure
Stable diameter after 30 days of pulsatile loading
Withstand more than 1,700 mm Hg pressure burst pressure
Complete coverage by ECs.
Biocompatible
No healing disturbances
Nontoxic
Nonallergic
No induction of malignancies
Minimally traumatic to blood compounds
Nonthrombogenic
Resistant to infection
Compliant: $4\text{--}8\%/ \text{mmHg} \times 10^{-2}$ ⁵¹
Flexible, elastic, without kinking
Resistant to myointimal hyperplasia
Easy processing
Adequate physical and chemical properties, mechanical durability: native arteries 5,000 mmHg, saphenous vein 1,680 mmHg ^{5,17}
Vessel diameters, wall thicknesses and lengths customizable to patient requirements
No need for special storage or preparation procedures
Easy to suture: 3 N ^{26,52}
Sterilization (radiation)
Optional
Capable of local drug delivery
Low costs

Despite the best efforts of many researchers to implement autologous and engineered vessels as replacement options, there continues to be failures with biocompatibility that account for the distinct features of biologic vessels [2]. Although ePTFE compliance is not examined during the BVM culture, future testing configurations with bifurcated and abnormal vessel constructions with vessel shape-movement, and volume capacity of vessels before and after culture periods could

provide insight into possible modes of failure of constructs based on the mechanical properties of a scaffold.

Looking to improve scaffold development based on geometry and material characteristics should improve the BVM model by making the assertion that the ideal scaffold will contain the physiological characteristics of compliance, biocompatibility, and porosity that will improve the development of a native endothelial layer within an ePTFE or natural polymeric vessel. The following sections will look at scaffold development as well as the general characteristics of scaffolds, and how they respond in static and dynamic culture environments.

1.2.5 Scaffold Development

The following sections describe TEVG approaches to scaffold development and incorporation of chemical constituents found in blood vessels. Without scaffold development, there would be little chance to solve issues that arise with compliance matching and maintaining endothelial stability and phenotype. There is no single preferred method of how scaffolds are prepared or processed, but present day studies are looking to create scaffolds that closely mimic the mechanical/biological features of native vessels in a laboratory setting while developing viable cellular networks.

1.2.5.1 Scaffold Development with Well-Plate and Electrospun Scaffolds in Static Culture Conditions

There have been certain types and methods of scaffold creation that have lent themselves to an increase in cellular adhesion and tissue growth rates. These studies

have been useful, specifically with understanding antithrombogenic modifications to vessels necessary to develop cardiovascular devices such as stents, grafts, and vascularized tissues [14]. Well-plate scaffolds have been a common method in TE studies of creating a semi-stable polymer mold in constructed wells to capture and cultivate mammalian cells in combination with usually a collagen or additional polymeric blend. Researchers in the Netherlands sought to understand the regulations and influence of an endothelium based on an extracellular matrix (ECM) composition. The mechanical properties of the tissue constructs were evaluated after creating an EC layer on rectangular 3D cardiovascular constructs using human venous cells seeded on polyglycolic acid (PGA) poly-4-hydroxybutyrate with EC media; within the studies the well plates proved to be a reliable method of a stable scaffold construction in the TEVG procedures [1].

The benefits of incorporating EC media to rectangular TE scaffolds revealed that a longer co-culture period between ECs, and myofibroblasts (MFs) had a positive outcome on cell retention when the cells were subject to shear stress [1]. To understand further significance of various ECM components on EC viability; polystyrene culture plates were used to compare the effect of angiogenic (AGF) and platelet growth factor (PGF) on remodeling the ECM statically *in vitro* [15]. This is based on the observation that ECs will upregulate junction and adhesion molecules after a long period of confluency [16]. After a week of culture, a confluent monolayer of ECs was found in both groups, so the process was maintained for an additional week to investigate the stability of the monolayer over time.

Throughout current TEVG studies, PGA, a biodegradable synthetic polymer was incorporated due to its ease in processing, and control of mechanical properties. However, biodegradable synthetic polymers suffer from poor cell adhesion, proliferation, and unwanted matrix synthesis [17]. On the other hand, natural polymers can replicate crucial features of their natural counterparts; such as surface free energy and hydrophilicity, and may improve cell adhesion [18]. Yet most natural polymers at the macro-size level do not have adequate mechanical properties to substitute as vascular tissue. However, natural polymers like silk-fibroin grafts have demonstrated excellent biocompatibility, controlled degradability, and versatile processing [19-21]. A realization of the benefits that these polymers possess has culminated to putting the vessels on the forefront of current TEVG projects.

Before moving into specific culture conditions of TEVG studies, it is worth mentioning electrospun tubular scaffolds. Electrospun scaffolds have provided versatility with the use of natural polymers to provide proper porosity and fiber length. Electrospinning technology has shown to be successful in blending different polymer materials at the nanometer level for tissue constructs, as demonstrated by natural collagen-PCL blends to recreate human skin [22]. Previous cultures with nanostructured PLGA have also shown elevated EC adhesion, proliferation, and ECM synthesis when compared to nanosmooth surfaces [7]. Electrospun scaffolds for example can range in wall thicknesses of 100 μm to 500 μm depending on the configuration of the electrospinner instrument [23].

Furthermore, the electrospun grafts have shown promising strength characteristics in terms of resistance to arterial pressure and comparable tensile strength, and most importantly the ability to support vascular cell function. Shown in Figure 4 is a static and dynamic culture comparison of HCASMCs on an electrospun silk fibroin scaffold [23]. Distinguishable from the image is the cell alignment, with respect to flow conditions for the static and dynamic states. Similarly, the TE lab has demonstrated the capability to develop a relatively controlled nano-fibrous topography that mimics the same quality of a native biologic vessel; making electrospun scaffolds more common in recent TEVG and BVM studies [24].

The effect of static culture environments on the mechanical properties of a TE vessel were also demonstrated in the latter portion of an EC culture utilizing well plate scaffolds. Constructs cultured in EC medium for 2 weeks had a low Young's moduli of 8 MPa versus 11 MPa after a culture period of one week, suggesting that a shorter culture period in EC medium is advised for a stiffer construct capable of bearing more load [1]. What was not demonstrated by this work was the use of dynamic culture settings to evaluate the endothelial formation in a TEVG under pulsatile flow.

In each of the studies above the versatility of scaffold creation allowed the researchers to vary the concentrations of natural and synthetic components within the scaffold matrix to theoretically create a more physiological accurate TEVG construct. However, in many cases the drawback with TE constructs that utilize static cell cultures is they have exhibited less development potential versus scaffold creation using dynamic culture conditions in TEVGs; this can be attributed to the lack of mechanical

stimuli found in a normal physiological setting [23]. Hence, it is worth introducing a few other TEVG studies using culture conditions that have shown to be more favorable for maintaining cellular growth, some of which has directly influenced the methodology of TEVG projects in the TE lab.

1.2.5.2 *Dynamic Shear Flow Environments*

As mentioned in the previous section, accompanying scaffold geometry and composition, the culture conditions that scaffolds experience is crucial for EC viability and endothelial development. Dynamic flow conditions generally improve mass transport and improve aerobic cell metabolism, as proven by the measurement of glucose consumption, and formation of lactate in the EC culture medium [23]. An investigation examined dynamic culture conditions to generate vascular grafts using electrospun polyethylene oxide (PEO), which was expanded on previous pilot studies that indicate EC constructs require more than just TE medium to proliferate and adhere [1, 23]. Also, studies have demonstrated applications of steady flow in a BVM model and TEVG respectively with electrospun scaffolds under similar methods, while using increased and steady flow rates through their culture systems [23, 24]. Furthermore, changes in cell viability and morphology were tracked and analyzed with the various setups to better understand EC morphology, immunohistology, and cell coverage within the implemented experimental conditions.

The findings that pertain to dynamic conditions in bioreactor systems will be recalled in hopes of distinguishing important observations, and any setup modifications used along the way. After culturing HCASMCs and HAECs in a closed-loop perfusion

system under increasing flow rates it was seen that there was random spreading and some detachment of HAECs along the lumen; however, significant HCASMC alignment along the direction of flow was exhibited on the luminal surface [23]. Moreover, it was concluded that the enhancement of tissue formation, ECM production, cell alignment and the retention of differentiated cell phenotype was induced under dynamic culture conditions in contrast to static controls [23, 25].

Exposure and conditioning of ECs at a dynamic flow setting showed tremendous potential within the TEVG studies described. At a low shear stress of 1.5 dyn/cm^2 SEM images revealed ECs oriented to the pulsatile flow direction and cell elongation [25]. Protein-modified stents exposed to a luminal flow of 15 mL/min showed a dramatically larger cell coverage compared to bare metal stents [4]. Although, earlier findings also revealed that proper cobblestone morphology of ECs was established at the same flow rate over a two week period on bare metal stents [6]. The cell elongation and typical EC protein expressions that was observed by the researchers warrants the idea that TEVG cultures under dynamic flow can lead to improved EC response and more accurate physiological TEVG models created in an *in vitro* setting [6, 21, 25, 26]. In many of the included setups the vessels were conditioned prior to cell incorporation to improve cell adhesion by conditioning the scaffold surface to better accommodate the ECs being introduced into the system [6, 25]. The benefits of these steps will be introduced to help justify more critical facets of the BVM protocol.

Static Culture

Dynamic Culture

Figure 4: A-D represent SEM images of HCASMCs seeded on the luminal surface for 7 days under static and dynamic condition while E-H represents the respective cultures at 14 days. Cell alignment (double white arrows) is seen along the flow direction (red arrow) for HCASMCs under pulsatile flow condition. Scale bars are: (A, C, E, G) 20 μm ; (B, D, F, H) 10 μm [17].

1.2.6 Vessel Conditioning

For many TEVGs created in an *in vitro* environment, preconditioning is required for a successful replacement graft or BVM. What remains missing in tissue engineering is the ability to develop TEVGs that closely resemble the native vasculature as well as the mechanical properties of biologic tissues *in vitro*. Having a functional tissue construct that one day possesses receptor-mediated signal systems will help reach a

more optimal replacement vessel; still, there has been no *in vitro* developed TEVG capable of smooth muscle contractility [27].

Major problems with commonly used synthetic grafts are the rigidity of the vessels and the subsequent inflammatory responses of introduced cell lines; this compliance mismatch with native vessels induces hyperplasia at the anastomosis which leads to variation between turbulent and low flow resulting in thrombotic stenosis or occlusion of the graft [8]. However, pre-conditioned grafts have shown to improve compliance of TEVGs as well as cell retention [27]. For example, cell adhesion has been improved by pre-conditioning ePTFE vessels with 15% FBS solution by capping the distal end of the graft and forcing the solution through the interstitial pores [28].

The maneuverability of preconditioning stages in the culture process can be valuable in developing new schemes within TEVG development; such as the static co-cultures of SMC-EC, a study that implemented a conditioning stage that was attributed to the increase of EC retention and nitric oxide production, a crucial signaling chemical [29, 30]. During another culture study pre-treatment of glass cover slips with gelatin and fibronectin coating was done prior to HUVEC incorporation and seen to improve confluency [31]. Consequently, improving ways of maintaining long-term EC viability through preconditioning steps are crucial for a tissue-engineered blood vessel models because of the dynamic capability of ECs to modulate normal homeostasis, and pathologic conditions by balancing antithrombotic and prothrombotic properties [26].

Some important observations and results from TEVG studies have been summarized to help describe some of the important methodologies and goals that have

had an impact on current TE fields. The subsequent few sections will explore cultivation techniques of HUVECs, and studies that directly used HUVECs to evaluate particular cell responses. To close out the chapter further insight into cell delivery methods with ECs and HUVECs will be drawn upon as well an overall summary of the aims and goals of the studies entailed within the project.

1.3 Isolation and Cultivation Techniques of HUVECs

Throughout this section, specific isolation and cultivation techniques of human umbilical vein endothelial cells (HUVECs) will be examined. Based on the ease of obtaining and passing HUVECs, HUVEC culture development onto synthetic and other polymer blends is becoming a more common approach in TE studies. Current biomedical engineering research using EC culture protocols in either a co-culture or isolated environment is worth further examination. Later, findings from these experiments and future focuses of HUVECs in TEVGs will be discussed.

1.3.1 Isolation Methods

Much of the attention thus far has been focused on general TEVG procedures and revelations that have led to the progressive development of more physiologically accurate TEVGs. Now the focus of TEVG experiments shift towards HUVECs in replicating an endothelium within a tubular polymer scaffold vessel. HUVECs have proved reliable in their ability to form a quiescent endothelium in BVMs and are a common EC cell type used because of cost and availability [32].

As the name suggests HUVECs are derived from donated human umbilical cord veins. After the umbilical cord is isolated it can be packaged and frozen for later retrieval of HUVECs and other cell types such as SMCs. The isolation of the endothelial cells from the primary vein of the umbilical cord has been standardized by biological practices, and will be summarized primarily from the technique demonstrated by Jaeger Davis of the Molecular Biology Department at UC Davis [33].

First, the umbilical cord is laid on a table and fresh cuts are made on both ends of the cord. Then a ½ inch gauge needle with a plastic sheath is inserted into the vein, the vein is located at the largest opening on the cord end while the smaller openings are arteries. A needle is clamped in position just inside the vein opening, and then a 20 cc syringe filled with hanks solution is attached to the needle. Hanks solution is pushed through the needle with moderate pressure; the Hanks solution flushes out red blood cells and other waste out of the vein. The process is repeated through the other end of the cord. After a few minutes the 20 cc syringe is removed and a 10 cc syringe is attached to the needle [33].

Moving forward the 10 cc syringe is filled with collagenase and pushed through the vessel until there is slow rhythm of droplet solution coming out the open end. The collagenase serves to rinse out any residual Hanks left in the cord and free the ECs. Once a droplet streak is observed the open end is re-clamped and filled with collagenase until moderate distention in the vein is observed. The cord is then laid back on a table and massaged gently to dislodge the HUVECs. This step requires sensitive

application of fingers to massage the cord; too much pressure will risk smooth muscle cell contamination. At this stage the cord is attached with a hemostat, needle, and syringe and are put inside a beaker of Dulbecco's Phosphate Buffered Saline (DPBS) and incubated at 37°C for 15 min. The DPBS serves as medium to keep the pH level within physiological range as well providing cells with water and bulk organic ions crucial for normal cell metabolism [33].

After incubation, the cord is taken out of the beaker and held over a 50 mL conical tube, the cord end is cut above the bottom clamp. All the outgoing fluid must be collected into the conical. The remaining collagenase is pushed through the cord and then the 20 cc syringe is reattached to the needle filled with Hanks solution. Once more the Hanks solution is pushed through vein with moderate pressure. Next the conical tubes are spun in a centrifuge at 1200 rpm for 5 min. Once removed from the centrifuge, the solution is aspirated except for the bottom 1-2 mL. The pellet observed at the bottom of the conical is now composed of some remaining red blood cells and the precious HUVECs. Now 5 ml of PHEC+ solution is added to the pellet and is flushed back and forth with a pipette until it has dissolved back into the remaining solution. This solution is transferred over to a T25 flask and incubated at 37°C at 5% CO₂ overnight [33].

The next day the supernatant solution is removed and replaced with fresh solution. If red blood cells are still present the solution is washed with M199 and PHEC and then incubated again in the same manner until a confluent plate of HUVECs is

observed. Once confluent, the solution is transferred into a gelatin-coated T75 flask, and once that flask is confluent the solution is separated into two vials and frozen for preservation and sterility assurance [33]. Finally, the HUVECs are ready for cultivation and experimental usage.

1.3.2 Characterizing Viable Culture and Growth Techniques

Cell culture techniques for HUVECs in TEVG methods vary based on slight differences in the types of medium-supplements implemented in specific research studies. Normally cell medium used in TEVG development are used to saturate the said scaffold(s) being used in the experiment with favorable growth reagents that improve cell attachment, viability, and minimize thrombogenic attributes [14]. Typically included growth supplements/medias include 1% penicillin/streptomycin, fungizone, L-glutamine, Fetal Bovine Serum (FBS), and M199; all agents are incorporated and directed towards maintaining HUVEC viability over long culture durations, and passages in TEVG studies including the current BVM model. Some agents are also directed at limiting microbial growth in the bioreactor/culture system [6, 25, 27].

A few essential factors play a key role when trying to analyze the success of a new culture technique implemented during the course of a TEVG study. A good starting point is asking what specific feature of a previous protocol is trying to be improved. Is the goal to compare the obtained results to a previous study under the same conditions? Are the types of cells being used in the research appropriate for the results being sought?

Over the next few sections, studies regarding culture media and supplement usage in HUVECs will be reviewed. It is important to note that the significant findings from the researchers also presented opportunities for conceptualizing and expanding on future TEVG studies specific to BVMs in the TE lab with regards to serum usages, media incorporations, and exposure times of culture media specific to EC cultures.

1.3.2.1 *Application of Different Growth Media Conditioning*

The media reagents and supplements that have been described thus far all have certain purposes and benefits in terms of cell proliferation and viability. However, specific publications have been aimed at whether substituting or eliminating specific culture media all together can have a profound impact on the cells ability to maintain viability within a population. Certain publications have claimed the development of a serum-free media for vascular EC and HUVEC cultures [34-36]. Also, various case studies have studied the long-term cultivation of HUVECs with the presence of serum-free medium versus prepared conditioned medium to observe the adverse effects of both styles of EC cultures [37].

The serum free medium utilized by researches in Germany was called TUD-1, a further development of a previously described medium optimized for growing HUVECs with IFs (intermediate filaments) as a basal medium [38]. What was unique about TUD-1 were the high levels of glutamine, serine, the inclusion of acetylcysteine, and phosphoascorbic acid.

Another key issue with non-serum cultures deals with the factors experienced by HUVECs and other types of ECs in slightly atypical culture conditions. With vessel modeling fetal bovine serum (FBS) was growth was compared after being substituted by patient-derived 2% AB human serum to establish a confluent HUVEC-Fibroblast co-culture in a 3-D spheroid culture. To evaluate EC network formation, CD31-positive areas were quantified; tubular network formation under human serum w/ incorporated EGM-2 media was significantly higher than in the 2% FBS control [39]. This implies that EC cultures were more confluent in EGM-2 incorporated cultures. ECs cultured in M199 over extensive passages have shown a different morphology, with irregular size and shape, compared to ECs cultured in EGM-2 [31]. Comparisons from the earlier studies indicate that an essential serum component in the normal media was not substituted by TUD-1 medium, and in the latter experiment by 2% FBS [37, 39].

Throughout expanding work it has been revealed that selective media may enhance the growth of different ECs, but the differentiated functions of the cells are quickly lost if high amount serum is added to the medium [31, 37]. With the use of selective medias it was observed that commonly used fibroblast growth media, resulted in lumenosis; the loss of the lumen formation within a TEVG [40]. This helped researchers continue further research into establishing the correct genes responsible of the lumen formation of native vessels.

Below are a few respective SEM images (Figure 5) of HUVEC morphology at the sixth passage stage, and HUVEC confluency using TUD-1 versus serum free media (Figure 6). Beyond the sixth passage, HUVECs cultured in EGM-2 maintained a

monolayer with distinct boundaries, whereas the cells in M199 appeared irregular in morphology.

Figure 5: SEM images of HUVECs at a P6 (sixth passage). In the upper figure (A), EGM-2 cultured HUVECs show homogeneous flat cells 20 – 40 µm in size, while the lower view of ECs cultured in M199 show cell size increased to 90 µm with no distinct cell boundary [41].

Although results are varied, these results indicate that alone, EC growth factors (ECGFs) and serums are not sufficient for culturing and maintaining HUVECs, and that the presence of hormones and other ECGFs are critical to maintain higher level cell passages [37, 41, 42]. Nonetheless, it was revealed that tubular network formation

under human serum was concentration dependent, with 2% AB serum giving the best results [39].

For a well-rounded perspective, repeated studies with similar protocol settings need to be executed to look at media impact on TEVG systems. It seems that certain combinations of culture medias and growth supplements has given the best TEVG results when ECs are used for blood vessel construction. The current BVM model has built on the use of certain culture media usages at different stages of the cell culture process to better assess the attributes of cell culture parameters.

Figure 6: A summary of HUVEC cultivation in TUD-1 and in serum-containing medium. The cultures were passaged every 4-5 days with reseeding at a cell density of 20,000 cells/cm². Each bar represents the final cell density before the culture was split as the arithmetic mean of three individual flasks; error bars depicts the SEM [37].

1.3.2.2 HUVEC Cultures

Revisiting the vital effect of mitogens and serum concentration on HUVECs, it was explained that EGM-2 maintained normal gene and phenotype expression and more viable HUVECs than M199 when cells were doubled over several passages. The researches in addition examined the expression of sICAM-1 by ELISA on the primary and 6th passages of HUVECs [31]. No significant difference in sICAM-1 concentration was found in primary cultures of HUVEC in M199 media and EGM-2. However, by the time the cells were passed to a sixth stage, concentration of sICAM-1 was decreased 4.2 fold in HUVEC cultured in M199 media compared to those cultured in EGM-2 media [31]. Conversely, when M199 was used as the bioreactor media in the shear flow effect study, population doublings occurred up to eight times without any adverse negative effects that could be attributed to the media alone [43]. While the main objectives of this study focus on consistent usages of cell medias during the BVM culture, it is interesting to note that the mentioned research initially cultured ECs in EGM-2, and then in M199 for the cell seeding and flow loading stage.

The cultivation of HUVECs has become more sophisticated in recent culture systems. What drives the continual pursuit of perfecting the cultural environment and maintenance of HUVECs is the inherent difficulty of preserving ECs when they are subjected to tissue engineering conditions. ECs used to create an endothelial layer into multilayer tissue structures in TEVGs are just a few of the *in vitro* and *in vivo* applications that are currently being pursued. Nonetheless, HUVEC protein expressions, morphology, and viability outcomes in specific environments still serve as

a solid representation/model of vascular ECs in general. It is for this reason and the ready availability of HUVECs that allows the cells to be utilized with respect to other types of isolated ECs. The following sections explore more specific TEVG applications using HUVECs.

1.4 HUVEC Development in TEVGs

Although the various protocols that exist for HUVEC cultures have similar techniques, the manners in which they are used in TEVG experiments will depend on the specific attribute of the cell being observed. A few practical fields that are well studied in tissue engineering HUVECs include cell morphology and phenotype analysis in various culture environments, endothelium formation with HUVECs in *in vivo* applications, the effects of different flow conditions on HUVEC adhesion on scaffolds, and co-culture development with other cell types. It is worth addressing the parameters used in these topics to supplement the previous sections and provide the reader with an idea of how different areas in TEVG development come together as a segue-way into the main objectives.

1.4.1 *In Vivo* Studies

Past and current research in scaffold development, isolation, and use of culture medias of ECs all play a role in proposed testing models surrounding TEVGs aimed at creating medical alternatives to severe health complications. To better understand the EC phenotype shift under haemodynamic and stromal microenvironments, Luu et. al compared neutrophil recruitment of HUVECs, human umbilical artery ECs (HUAECs),

and human coronary artery endothelial cells (HCAECs) after exposure to static and dynamic culture [42]. A further response of the study comes from the lack of current understanding of the rare combined effects of stromal haemodynamic factors [44]. It was for this reason the ECs were tested to observe any differences among the cell lines in their ability to support adhesion of flowing neutrophils, and whether this depended on the culture media and or conditioning by shear stress [42]. The significance of the results from shear and static environments along with comparable research on stent materials will be brought to attention in the subsequent section.

It is simple to picture the near limitless applications that HUVECs serve for a variety of TEVG research in the tissue engineering and the medical field. The antiangiogenic therapies involving HUVECs and vascular endothelial growth factor (VEGF) upregulation is another example of an *in vivo* application using past research to better understand the potential of a therapeutic protein (VEGF) for antiangiogenic therapy [45-48]. Also, the concept of incorporating HUVECs with microencapsulation scaffolds, and the timed release of accurate dosages of angiogenic inhibitors encompass newer forms of creativity behind *in vivo* scaffold technology [49]. Of course these mentioned studies would not have been possible without the culmination of previous biochemical and culture testing of ECs in similar physiological environments. Hence, in the next passage, HUVEC TEVG applications in flow environments, as well as validation of perfusion systems for high cell density cultures will be presented.

1.4.2 HUVEC Development and Response in Static and Shear Environments

The studies mentioned thus far have used HUVEC cell lines to gather research on the adaptive responses of the cells in various culture environments. From some of the initial studies presented it can be suggested that the type of shear imposed flow on HUVECs will have just as large of an impact on correct cell phenotype expression than the culture duration of HUVECs. To demonstrate the effect of gradually graded shear stress on HUVEC-seeded grafts, an electrospun PLCL tube was preconditioned by forcing 0.2% neutral type collagen I through the micropores of the tube [25]. In a different HUVEC culture, a fibrin gel was created to examine pulsatile flow vs. static culture. Chouinard et. al added fibrinogen to a HUVEC cell suspension to obtain a transparent and homogenous fibrin gel [43]. These gels were attached to an in-house bioreactor and examined for HUVEC growth in a culture media perfusion with an applied flow rate of 70 mL/min with a pulsated 40/20 mmHg static pressure [43].

Within the HUVEC-seeded SDVGs there were significant morphological differences between the static and shear stress controls [25]. HUVEC-seeded tubes in the static group demonstrated almost identical morphological features irrespective of culture time. Most of the luminal surface was covered with polygonal cobblestone shaped HUVECs as exhibited in Figure 7 (A-D) [25]. This is an indication of proper EC morphology shift observed in EC mono-layering with correct shear stress characteristic resistance [50, 51]. Under similar growth conditions, at an initial concentration of 1×10^6 cells/ml, both dynamic and static cultures revealed few living HUVECs on the top of the gels in static culture, and on the bottom of the thick gels only dead cells were present

[43]. Conversely, in samples cultured only under pulsatile conditions, HUVECs were found homogenously distributed within the cell and tissue culture chamber with very few dead cells [43]. This data reaffirms some of the benefits of shear imposed conditions on EC cultures.

The assessment from each study showed that hollow fiber bioreactor chambers under pulsatile shear induced conditions produced and maintained the best HUVEC morphology, viability, as well as nutrient concentrations and transport in the extracellular region [25, 43]. Further cell structure observations seem to warrant the claims by the researchers. A key cytoskeletal component actin, was observed as being oriented randomly as observed in Fig. 8 (E) and VE cadherin, a principal cell-to-cell adhesive protein, was found at adjacent contact regions during gradually applied shear stress on HUVEC vessels in Fig. 8 (F) [25].

Figure 7: Morphological images of the luminal surface of HUVEC-seeded graft under 24 hours of static culture. Images A and B are SEM snapshots, while C and D are CLSM images with DiO-labeled cells; E shows actin fibers and VE-cadherin, F [24].

There were also some further revealing comparisons among static and dynamic HUVEC cultures in the discussed studies. It was apparent from the system that the available oxygen concentration in static cultures resulted in lower cell numbers after two days versus the dynamic culture setup; cell proliferation numbers were much higher with fibrin gels seeded with HUVECs [43]. What is important about Inoguchi's work is the demonstration that a stepwise increase in graded shear stress resulted in full cell retention with cell morphology and alignment consistent with native ECs; furthermore, shear stresses with the appropriate magnitude may be the key to enhance the synthesis

and secretion of bioactive substances such as endothelial cell nitric oxide synthetase (eNOS) [52, 53].

A second key finding from Luu et. al added weight to the idea that changes in shear stress as well as stromal factors contribute to variations in EC gene characteristics in different locations of vasculature [42]. Primary HUVECs cultured for 5 days in static cultures exhibited a different phenotype from cells isolated freshly from the umbilical vein, as observed by expressions of inflammatory related genes [42]. With long term HUVEC cultures, deposition of a basement membrane was associated with changes in neutrophil migration induced by TNF- α , and changes in matrix proteins influencing EC response to shear exposures [54, 55]. Taking this into account, results indicate that site-dependent responses to inflammatory stimuli may arise from changes in flow conditions and stromal or cell tissue structures in local vessels [42].

While most of the findings support the idea that shear flow helps the overall development of an endothelium in TEVG constructs, the continued pursuit of HUVEC culture evaluation on various materials in either static or dynamic environments continues today. Since the ground work of what desirable characteristics a recreated endothelium should possess regarding morphology and phenotype expressions, the following section will briefly describe studies that analyzed HUVEC viability on various stent metals with different culture and media exposures.

1.4.3 HUVEC Development on Stent Materials

HUVEC studies with stent materials have assisted the evaluation of material selection of stent devices, and learning how to tailor the device to the surrounding endothelium of a native blood vessel. Previous reports have showed that TiN and TiO₂ - have good biocompatibility characteristics with other types of cells [56, 57]. Here, the effect of cell viability on stent metals was examined as well as stent modification on HUVEC profiles under static conditions.

It was noted that several crucial stent features such as thickness, edge angle, drug coatings, and surface grooving affects the injury and regeneration capabilities of HUVECs [58, 59]. However, in an attempt to better characterize HUVEC phenotype on stent metals, HUVECs were seeded on metallic sheets and placed in a 35 mm tissue culture-treated polystyrene dish filled with M199 culture medium containing fetal cattle serum, containing several previously listed growth reagents [60]. Furthermore the goals of the study evaluated eNOS, and VWF with HUVEC expression, along with other EC markers represented below, and found that HUVECs seeded on the metallic sheets regardless of the coating had higher values of cellularity, but lower expression levels of Cx43, eNOS and VWF (von Willebrand Factor) [60].

Yet, because TiN and TiO₂ coatings did not change the down regulation of Cx43 gap junctions (which is associated with a decreased expression of eNOS), suggests that the overlying of endothelial cells had a functional defect [60]. Although this hypothesis was not analyzed here, the possible detriment of a static environment of HUVECs may have accounted for the clustering of ECs on metallic materials and lower

expression levels of eNOS and VWF. However, a proposed study of dynamic cultures of HUVECs on stent materials may have been worth investigating by the researchers to determine if any differences would have occurred at those chosen seeding densities.

Figure 8: A growth index of HUVECs on metallic materials. Comparisons are made within experiments using the same starting seeding density. $p < 0.05$ and $p < 0.01$ compared to each of TiN, TiO₂, and control (YEH).

Nonetheless, the finding was still significant because the down-regulation of Cx43 gap junctions is closely linked to endothelial dysfunction [61]. Since it is known that endothelial dysfunction predisposes the vascular wall to inflammatory cells infiltration, platelet aggregation, and SMC migration and proliferation; the presence of endothelial dysfunction in stented arteries can lead to unwanted complications such as in-stent thrombosis and restenosis [62-64]. Also, what was not mentioned in the study was the effect, if any, the inherent surface roughness of each metal and polystyrene

control had on the overall adaptability and behavior of the HUVECs. What is summarized in the following passage are specific cases of scaffold surface roughness and modification of polymers used in TEVG studies with HUVECs.

1.4.4 Effects of Scaffold Surface Roughness

The evaluation of surface roughness and material quality along the inner lumen wall of polymer scaffolds is another important facet of improving the endothelium in potential TEVG/BVM setups. Scaffolds modified at the surface level with manipulated polymer frames have exhibited excellent EC response in various studies, but still, it is a relatively new field of research. In order to have a general grasp of cell adhesion potentials on different surface roughness degrees, these studies looked at pinpointing any differences in HUVEC expression within various characterized scaffold surface properties.

Previously, the development of ECs on various PMMA surface roughness was concluded to show a larger degree of surface roughness may have positive effects on EC adhesion due to the triggering of sub-confluent cells to secrete extra-cellular proteins which allow anchorage of cells to their substratum [65]. Another source reported that the degree of roughness of a titanium metal surface can modulate the product of cytokine and growth factors of vascular chicken embryo ECs, but with reduced cell numbers [60]. However, the tracking of cell adhesion within *in vitro* models was the critical aspect of the following studies.

Modifying scaffold topographies even at the nanometer scale has shown clear benefits to transanastomotic cell migration and the recruitment of biomolecules for

circulating ECs and EPCs [14, 66]. From previous reports it was noted that various factors of scaffold composition can also influence cell affinity, such as surface morphology and chemical composition [66]. When looking back at the cell adhesion and growth of HUVECs on PU-PEG films, statistically rougher surfaces were better than that of smooth surfaces for both GRGD grafted and un-grafted surfaces, respectively; increasing roughness of a scaffold surface even at 10-100 nm scales was shown to enhance HUVECs adhesion and growth on the surface of GRGD surfaces [67]. In addition, research has revealed that small active sequences in ECM proteins exert an influence on cell-matrix interactions, so structural atom modification could be an effective strategy for promoting the cell affinity of biomaterials [68].

Liu et. al discussed the role of these ECM proteins and the influence on cell-matrix interactions, by including the Pro-His-Ser-Arg-Asn (PHSRN) peptide in HUVEC adhesion and growth to study modified polymers in vascular synthesis development [32]. The PHSRN and GRGDS containing polymers responded well under culture incorporation of HUVECs. The polymers that were synthesized containing these peptides (P-PN5 and P-GS5, respectively) had similar results of HUVEC adhesion and proliferation to PLMA ($p < 0.05$) as seen in in Figure 9 [32]. However, a steady increase in the cell confluency on P-PN5 was found from day one to seven when cell seeding was done at 4×10^4 cells/mL in accordance with previous literature [69].

Figure 9: HUVEC number on polymer modified materials after 1, 3, 5, 6, and 7 days. Cell seeding density at 4×10^4 cells/mL. *p < 0.05 [62]

In review of the studies of adhesion and proliferation, results indicated that certain compounds and peptides such as PHSRN are capable of enhancing cell affinity of the materials. When PHSRN was modified to a longer hydrophilic linker such as PEG, the longer PEG chain could mask the peptides available to the cells [70]. Although, modified hydrophilic peptides attached to the hydrophobic polymer backbone may allow the peptides to be more accessible for the binding of EC surface receptors [32]. Also, stated throughout the studies was that rougher surfaces at a very specific

and controlled degree became beneficial to cell viability over long culture durations. This interpretation is relevant to BVM setups that are currently utilizing new conditioning methods, and polymer creations to improve EC adhesion.

So far topics of *in vitro* HUVEC development and their use in TEVG experiments have been discussed. Although this study is also done in an *in vitro* setting, it is important to remember that the eventual goal of BVM modeling and tissue regeneration is testing clinical arterio-related devices for eventual *in vivo* applications. If there is not a high level of vascularization in the transplanted grafts, the majority of cells fail to survive the early post-transplantational phase [71]. In the following discussion a few relevant applications of HUVEC-seeded grafts are compared by media parameters that influenced the success or failure of TEVGs in a long-term culture.

1.4.5 Growth Factor Influence in HUVEC Cultures

The serum-free trials of HUVEC cultivation mentioned previously will be examined with more focus on the direct impact of HUVEC confluency and phenotype expression in various setups. The serum-free trail discussed in section 1.3.2.1 resulted in two distinct HUVEC morphologies when TUD-1 (a non-containing serum) media was used versus conventional serum medium. HUVEC shape in TUD-1 resulted in an extended elongated formation while the serum containing culture shows an expected prominent cobblestone pattern with the final cell densities of both cultures between $0.8\text{--}1.1 \times 10^5 \text{ cells cm}^{-2}$ [37]. Interestingly enough there was a difference in the appearance of normal endothelial markers such as the von Willebrand factor among the

implemented growth medias, where TUD-1 media showed slightly increased vWF expression.

With respect to the influence of serum usage on passage rates, the serum free media allowed for up to 20 cumulative population doublings without significant differences in final cell density compared to controls with HUVEC cultivation under serum media [37]. These advantages seemed to reduce the overall cost and interjection of biochemical components to the cell culture, a possible benefit that needs more investigation.

Up to now the emphasis of HUVEC culture and TEVG procedures have summarized important attempts in the tissue engineering field to better characterize the behavior and reaction of EC morphology, and roles within various *in vitro* and *in vivo* environments. A major contributing factor to the success of these studies has to do with cell engraftment or the execution of delivering the HUVECs along with other vascular cell types into the scaffold vessels utilized. The methodology of incorporating cultured cells onto scaffolds will be discussed in an attempt to optimize the process of cell sodding during the methods portion of Chapter 2.

1.5 Methods for Cell Delivery onto Scaffolds and TEVGs

There is not one single superior method of delivering cells onto a scaffold graft. A host of cell seeding and sodding techniques will therefore be discussed prior to the main objectives of this study.

1.5.1 Cell Sodding/Seeding Techniques

Cell sodding requires the input of the correct number of cells at a high population base into a natural or synthetic host vessel. At this point the cells have gone through a desired passage or population doubling(s) that have brought the culture to a confluency that requires no additional time for the cells to divide once they have been transplanted to the host graft [72]. However, cell division may occur within the scaffold structure itself during the culture duration of the TEVG.

Cell seeding is a process that uses fewer cells initially, meaning that at the deposition stage onto the graft there is still time required to allow for the cells to divide and proliferate further, this is the key difference between the two techniques. A commonly used technique, termed static seeding or sodding, consists of pipetting a concentrated cell suspension to a porous scaffold; leaving the user with less control and standardization in the process [72].

With respect to silk-based vascular grafts constructs, cell seeding methods have utilized HCASMCs by cell injection into the lumen of the scaffold from one end using syringes [23]. Once the bioreactor openings were closed after being filled with smooth muscle CM, the seeded scaffolds were placed on a roller apparatus to promote homogenous cell adhesion on the scaffolds (Figure 10). In a static cell seeding trial, decellularized vessels were seeded with VSMCs, with the most optimal conditions resulting from increasing the cell concentration value to 50×10^6 cells/mL, while the scaffolds were submerged in the culture medium [27].

Figure 10: A dual-loop bioreactor used in TEVG cultures. HCASMCs and HAECs were sequentially seeded onto the luminal surface of the tubular scaffolds under pulsatile flow [27].

Cell encapsulation, a more controlled form of capturing cells through seeding injection methods, has shown suitable potential in TEVG applications. The concept itself literally revolves around capturing the target cells within a biocompatible polymer that can adequately maintain structure as well as cell viability. Hahn et. al used a modified PEG polymer hydrogel to capture cells 10T-1/2 mouse smooth muscle progenitor cells at desired cell concentrations [73]. The earlier investigation of HUVEC neutrophil recruitment used seeding methodologies to capture HUVECs on rectangular glass capillaries which were coated with collagen/gelatin [42]. With an introduction of cell seeding-sodding methods, the sodding methods presented in chapter two will use similar injection schemes in a BVM specific environment.

Within the course of literature found in TEVG studies, cell seeding is the specified term for most cell delivery processes while cell sodding techniques seem to be less used. Hence, the type of study itself can play a role into which method would be

more beneficial. Many of the studies start with an initial specified concentration of cells and look to see if cell expansion takes places or not. Contrarily, in BVM studies the goal remains to see if adhesion and lining can be established through sodding methods; cell pressure sodding was performed using HMVECs in a BVM study by restricting the luminal flow path and creating an outward transmural direction of the cells into the prepared ePTFE vessel [6]. This technique in essence forces the incorporated cells to go one direction; which is outward or transmurally through pores of the fixed scaffold.

The various initiations of cultured or modified mammalian cells into TEVGs as described, can take many forms. Whether the TEVG study looks to compare static vs. dynamic flow settings in EC monolayer development, or various preconditioning steps, the final outcome requires repeated studies to examine if a correlation of a specific parameter or technique relates to the success or failures of a desired vessel.

1.5.2 HUVEC Sodding Trials in ePTFE Scaffolds

Since ePTFE was the material used in the BVM studies moving forward, it is worth taking one more peripheral view of the TEVG studies that have used ePTFE scaffolds at the time of EC/HUVEC injection. In this regard, HUVECs were transferred to living tissue conduits grown in the peritoneal cavity of dogs subjected to static cultures for two days followed by dynamic exposure for two days at 1.5 dyn/cm^2 which seemed to improve the monolayer confluency and resistance to shear stress [74]. To follow up on earlier techniques, studies focused on HUVEC morphology investigated shear stress and modified serum environments [25, 31]. What differentiated the two

studies are the techniques of the cell incorporation. An electrospun 6 cm PLCL tube was immersed in 70% ethanol overnight for sterilization and prewetting, and then prior to cell incorporation the tube was incubated for 30 min at 37°C to allow gel formation; the process was repeated four times as the tube was rotated horizontally 90° into a cell rich solution [25]. A standardized version of rotating the scaffold vessel before cell injection was proven to be successful in maintaining a confluent endothelium. Similar to cell sodding methods, cell plating has utilized the protocols looking at HUVEC proliferation and growth.

1.6 Summary and Aims of the Thesis

So far a review of pertinent components in TEVG methodology has been explored. Use of HUVECs *in vitro*, including isolation and cultivation practices, alongside research in HUVEC-based TEVGs was summarized. Cell seeding/sodding practices were detailed from a host of tissue engineering experiments that involved the use of a particular scaffold graft and culture mediums. The rest of this project will focus on the experiments that have drawn principles from these fields to attempt a successful tissue engineered BVM; in addition to previous research, quality control techniques will be implemented to assess preliminary lab contamination risk, with a goal of standardizing the performed protocols.

Moreover, the overall objective of this project is to improve BVM construction, and the consistency of endothelial monolayers constructed with HUVECs under peristaltic flow conditions inside a perfusion bioreactor system. Close observation of

HUVEC adhesion, coverage, and morphology will take place on the post analysis assessment of the BVMs at the conclusion of each study.

The first study will start with a BVM protocol for vessel development that was established much earlier in the tissue engineering lab. First, six BVM models will be cultured to examine if proper cell adhesion develops on the lumen of each ePTFE and to further review the changes of endothelial growth for the three culture durations. After cell coverage is observed from multiple grafts, the latter setups will look to focus more on system parameters. Adjusted model parameters will be tracked to see what effect they will have on the endothelial formation. Changes in the luminal endothelium with and without the application of media flow will also be discussed, and compared accordingly based upon the results. Through BBI and histological staining the differences in overall cell coverage and endothelium formation will be reviewed for each vessel.

In the second part of the project, microbiological assessments of the BVM culture will be made by examining the sterility of the surrounding tissue engineering lab environment. This goal of this part of the project is to track any microbial growth that may be growing around the cell culture area and hence be detrimental to the overall BVM development. Further reasoning and limitations behind the microbiological testing will be examined in detail in the coming sections.

A summary of the work and experimental findings will be presented in the conclusion chapter. All significant information regarding the initial BVM setups and later

microbiology experiments will be presented in a manner as to present the reader with an idea of how the BVM model was influenced from the various parameters within the BVM setups, including discussion on the possible long-term benefits from sterility assurance tracking and controlling microbial growth through improved culture practices. Future insight into the field will be explained in such way as to provide potential endeavors in the BVM field to help improve how the best physiological BVM replica can be created in the tissue engineering lab.

Chapter 2: Blood Vessel Mimic (BVM) Set-Ups Over One, Three, and Seven Days

2.1 Introduction to Blood Vessel Mimics

The concept of reconstructing the essential features of a tissue engineered blood vessel has made enormous strides in the last decade from the evolution of the research areas that were mentioned in Chapter 1. As scientists continue the pursuit of an ideal blood vessel, the testing provides a way to assess the development of an endothelial lining; from the preparation involved with experimental trials, and the post analysis results that reflect the goals of the BVM concept.

2.1.1 History of Blood Vessel Mimics

While the desired outcome of the BVMs in this study is to obtain an appropriate endothelium within a biocompatible ePTFE scaffold, the bigger goal of creating BVMs in the tissue engineering laboratory is to create ideal physiological environments to study intravascular device interaction with a TE blood vessel. Previous BVM research demonstrated the culturing of HUVECs onto 4-mm-ID ePTFE grafts to establish an endothelial cell lining to assess a stent-intima interaction and co-culture development with SMCs [24, 75].

Knowing that past BVM trials have shown signs of successful EC deposition along the luminal lining leads to inquiry on how will HUVEC cells behave in a modified setting, and how consistent the endothelium can be made in these environments? Since there are multiple culture parameters, one of the main focuses for the BVM

setups throughout this study was to begin to track any alterations to the protocol that may have affected the overall HUVEC coverage during the BVM culture period(s). This chapter summarizes important facets of three different BVM studies and the results associated with the BVM setups. Study one will be summarized independently, with more specificity described in the methods as to provide the major overall aspects of the processes involved. Studies two and three are presented more briefly due to their similarity in post analyses, with highlights of the methods, and differences that were noted in the setup. After the results of each study are examined, a summary of the experimental system will be presented and discussed, as well as what adjustments were suggested for future studies.

2.2 Materials and Methods

2.2.1 Materials and Methods for Study One

Human umbilical vein endothelial cells (HUVECs) were obtained at passage 1 level from Lonza. The HUVECs were frozen and preserved in a HC34 Taylor Wharton nitrogen (N₂) gas tank (Figure 11). After HUVEC transfer into the EC media, the T75 was put back inside the Shel Lab incubator (Figure 12) at a 37 °C, 5% CO₂ conditions until the following day for appropriate cell passage.

Figure 11: A HC34 Taylor-Wharton N₂ Tank for the preservation of various HUVEC cell populations.

Figure 12: Shel Lab Incubator operating at 37 °C and 5% CO₂.

The HUVECs were previously prepared by Dr. Kristen Cardinal to 4 population doublings. To expand the cell population to a 5th passage (P5), the prepared HUVECs at P4 (4th passage) were transferred between T75 culture flasks. The cells were observed under an Olympus CKX31 Optical Microscope (Figure 13). Initial confluency was recorded at 40 percent. The confluency describes the relative percent coverage of HUVECs on the observed area by the optical microscope. During the follow up passing stages the P5 HUVECs were ultimately passed to P8 with a final expected cell count of 10×10^6 cells per T225 flask. Six total BVMs were constructed from the P8 HUVECs. Detailed protocols of each passing stage are presented in Appendix A.

Figure 13: CKX31 Optical Microscope used to track HUVEC confluency and observe morphological conditions of HUVECs during the cell culture duration.

To obtain proper sterility assurance for the BVM system all bioreactor materials, tools, scaffolds, and tubing (Figure 16-17), were either steam sterilized or passed through an ethylene oxide (EtO) gas sterilization by the Cal Poly Vet Clinic.

Figure 14: A media reservoir assembly consisting of a series of connecting silicone tubes that feed into the reservoir chamber.

Figure 15: A bioreactor chamber with attached flow valves.

For steam sterilization the appropriate materials were placed inside a M9 Ultraclave. The machine was adjusted to 30 minutes of steam sterilization process and an additional 30 minutes for drying (Figure 16-17). After the sterilization stage all sterile culture materials were ready for vessel preparation and BVM setup.

Figure 16: Here the M9 Ultraclave is closed and operating at the conditions described above.

Figure 17: The M9 Ultraclave in open position; a media flask covered in foil wrapping with water level present on side.

To adequately prepare for the BVM culture, ePTFE grafts, 3.5 cm long and 3 mm in diameter were used as the scaffold. The graft material was mounted onto barbed fittings and tied using silk suture around the edge of the bioreactor fitting for proper insulation during the denucleation and BVM culture stages.

As a preconditioning exposure, the denucleating process helped remove trapped air from the interstices of the ePTFE grafts. 10 ml vials of 70% and 100% ethanols were prepared for the grafts, along with 10 ml vials containing degassed conditioning media (CM) that was prepared in advance. CM helped aid the scaffolds with prior

protein exposure while seeking to potentially improve cell adhesion once HUVECs were sodded to the ePTFE scaffold during cell injection. Ingredients for culture medias used in the BVM setups are listed in Appendix I.

Through the applied protocol (in Appendix A) HUVECs were sodded on the ePTFE vessels and cultured in a perfusion bioreactor system. Two BVM vessels each were cultured at the one, three, and, seven day points. Illustrations of the bioreactor systems under the laminar flow hood and Shel culture incubator are depicted in Figures 18– 20.

Figure 18: A view of the Bioreactor setup underneath the hood during the scaffold priming stage. Troughs are located at the inlet and outlet of the bioreactor ports. ePTFE scaffolds were primed with conditioning media.

Upon completion of the respective cultures, each BVM was fixed and preserved in histochoice solution at one, three, and seven days for image analysis. Peristaltic flow rates were increased accordingly at the three and seven day takedown points for the remaining BVMs.

Figure 19: Multiple views of the completed BVM setup, including: the peristaltic pump with 8 roller trays, the 6 BVM bioreactor setups along with 6 media reservoirs composed of silicone tubing and thin pump tubing.

Figure 20: Each bioreactor system is configured with 1 respective media reservoir. Here the system is operating at 14 rpms.

One week after the original BBI preparation, the vessels were removed from 10 ml vials for image analysis with a fluorescent microscope. The microscope used a filter

to portray a wavelength of fluorescent light to correctly reflect HUVECs that were tagged with BBI along the BVM lumen. A complete protocol of the BBI image analysis can be found in Appendix G.

The histology staining protocol was specifically carried out as described in Appendix D. In summary, just prior to the series of staining procedures the vessels went through a few crucial steps of preparation. First, each of the six halves of the BVMs was embedded into wax blocks. The wax blocks were sliced using a microtome at 7 μ m sections and placed onto glass slides (see Appendix J). Once an adequate number of slides were prepared for each of the BVM cross sections, the slides were ready for H&E staining.

2.2.2 Materials and Methods for Study Two

For study two, cryopreserved P8 HUVECs were used as the initial cell passing point. An included adjustment was the preparation of culture flasks containing prewarmed HUVEC media at 37°C. Allowing the EC media to warm up to physiological temperatures allowed critical proteins to coat the flask surface to improve the adaptability of HUVECs upon transfer to a culture flask.

One passing stage was sufficient for the second BVM study since only one vessel was being incorporated with HUVECs. The HUVECs at the P8 level was sufficient to use after expanding the HUVECs to P9. Furthermore, the HUVECs were transferred into the T75 and put back into the Shel Lab incubator until the first passage was performed.

The BVM culture was carried out through a similar stepwise process used in study one that is detailed in Appendix B. However, study two sought to examine the parameters within the culture process of a static BVM setup with the goal of improving the HUVEC lining adhesion through standard cell sodding techniques, and tracking adjustments amongst the parameters of the culture setup. Specifically, in study two, the BVM was cultivated for 24 hours at static conditions.

2.2.3 Materials and Methods for Study Three

For the third BVM study, P4 HUVECs were utilized to create an endothelium in four ePTFE scaffolds. Following standard cell passage methodology, the HUVECs were passed from a P4 all the way to P7 by at the time of cell sodding. Two sets of BVMs were cultured under different flow conditions for one day durations. Specifically, 2 BVMs were exposed to increasing flow up to 15 RPMs for 24 hours, while the other two were cultured at a constant 15 RPM steady flow condition. The details of the BVM protocol for each set of vessels are described within Appendix C. Cell density counts and image analyses were performed as described previously.

2.3 Results and Observations

2.3.1 Results from Study One

In study one; six BVMs were constructed with various sodding densities prior to final incubation at 37°C, 5 % CO₂. To help characterize endothelial cell sodding densities, the expected cell densities were determined just before cell sodding onto the ePTFE scaffolds. This property of the cell culture was based on a few factors including

original P5 HUVEC population rates, cell confluency observations throughout the vessel setup, and final cell population. The surface area of the ePTFE scaffold was also taken into account to calculate the expected number of cells that were introduced into the BVM system at the point of cell sodding. The surface area of the graft was found from the equation, *Surface Area (SA) = $2\pi r * L$* , where r is the radius and L is the length of the ePTFE graft; 1 cm of the initial scaffold length was subtracted from the total length of the graft that was not exposed to the cell sodding regions. Confluency values are presented in Table 2 as well as Appendix K.

Table 2: Summarizes the range of cell confluency and cell sodding densities for each of the BVMs in study one. A greater confluency was observed for the later cultured vessels; including vessels E-F.

	Cell suspension	Surface Area	Cell Confluency	Sodding Density	Culture Time
Vessel A	10 X 10 ⁶ (cells)	2 π .15*2.5 = 2.36 cm ²	70%	3.0 x 10 ⁶ (cells/cm ²)	24 hours
Vessel B			70%	3.0 x 10 ⁶	24 hours
Vessel C			80%	3.4 x 10 ⁶	48 hours
Vessel D			80%	3.4 x 10 ⁶	48 hours
Vessel E			100%	4.2 x 10 ⁶	1 week
Vessel F			100%	4.2 x 10 ⁶	1 week

The images presented illustrate cell coverage along the vessel lumen. The scarcity of HUVECs observed on the lumen from study one made it too difficult to discernibly calculate cell coverage values.

2.3.1.1 *BBI Imaging*

2.3.1.1.1 Vessel A, 24 - HR at 3.0×10^6 (cells/cm²)

Figure 21: The proximal-middle region of vessel A (24 hour culture) is shown. Very few cells appear to be present from the lack of cell fluorescence seen on the cross section.

2.3.1.1.2 Vessel A and B, 24 - HR at 3.0×10^6 (cells/cm²)

Figure 22: Here, the middle-distal region of vessel B is shown. Not many cells were observed throughout the various regions B.

Figure 23: A similar viewing of vessel A at the distal region of the graft with respect to flow.

2.3.1.1.3 Vessel C, 72- HR at 3.4×10^6 cells/cm²

Figure 24: Vessel C cultured for 72 hours. Several faint cell type specs appear to fluoresce in the mid region of the vessel. Still, it is hard to conclude the presence of any established cell layer based on this BBI image.

2.3.1.1.4 Vessel D, 72 - HR at 3.4×10^6 cells/cm²

Figure 25: Vessel D cultured for 72 hours. Similar to Vessel C, few cells appear to fluoresce visibly in the mid-distal region of the vessel.

2.3.1.1.5 Vessel E, 1-week at 4.2×10^6 cells/cm²

Figure 26: Vessel E shows a mid-proximal image of the lumen surface. A possible series of cells fluoresce in the upper left area of the image.

2.3.1.1.6 Vessel E and F, 1-week at 4.2×10^6 cells/cm²

Figure 27 A mid-distal shot of vessel E confirms some presence of HUVECs, however the count was too low to obtain an accurate cell average count based on the viewing area.

Figure 28: Vessel F shows minimum cell coverage on the distal end of the graft.

2.3.1.2 Histology

2.3.1.2.1 Vessel A and B, 24 - HR at 3.0×10^6 (cells/cm²)

Figure 29: Vessels A at 4x magnification. HUVECs are not Vessel B at a cell sodding density of 2.5×10^6 cells/cm².

Figure 30: Vessel B at a cell sodding density of 2.5×10^6 cells/cm². No attributable lining was present from the above image. Vessel A observed at 4x magnification.

Figure 31: Left proximal section of Vessel A at 10x.

Figure 32: Right proximal view of Vessel B at 10x. Although pinkish globules appear along the lining, it was still inconclusive to say any endothelial layer was present on the graft.

2.3.1.2.2 Vessel C, 72- HR at 3.4×10^6 cells/cm²

Figure 33: Here a left angle proximal view of the lumen cross section of vessel C with a cell lining development.

Figure 34: Vessel C at 4x shows further structured lining indicated by the presence of a red stained strip of cells alongside the right side of the mid-proximal area.

Figure 35: Another view of Vessel C from the center region of the vessel cross-section. Here cell lining is evident and points to the consistency of the HUVEC layer that was established in this section of the vessel.

Figure 36: Vessel C at 10x, the thickness of the lining can be more easily differentiated from the ePTFE vessel.

2.3.1.2.3 Vessel E and F, 1-week at 4.2×10^6 cells/cm²

Figure 37: Vessel E cultured for 1 week with a shows consistent cell lining along what remains of the left proximal end section at 4x.

Figure 38: A separate isolated section of the top proximal area of Vessel E shows little sign of cell deposition along the lumen.

Figure 39: The arrows on Vessel F show penetration of HUVECs into the ePTFE interstices.

Figure 40: No presence of cells is seen on a separate cross sectional view of Vessel F.

Figure 41: On a separate slide, Vessel F at 4x shows a top-proximal view covered by a thin endothelial lining along the lumen area.

Figure 42: A right centered proximal view of Vessel E appears to have an endothelial lining consistently down the cross section.

Figure 43: Shows vessel F at 10x magnification with arrows pointing toward endothelial lining down the lumen.

Figure 44: Vessel E cultured at 10x indicates cell stacking evident among the middle region.

2.3.2 Results from Study Two

To characterize the cell seeding densities in study two, careful observations of cell confluence were made on setup day; 60 percent confluency was noted for the T225 culture flask for vessel 1. As previously calculated in study one, the expected number of viable cells incorporated into the vessel prior to the BVM culture was determined. Since a clearer amount of cells was seen from the BBI images, the cell density of cells per cm^2 was calculated along the lumen of each sample.

Since the study only utilized one BVM, the following images cover the cross sectional regions of the single vessel that was developed. The order of the imaging is presented in order of the media flow into the bioreactor and subsequently through the tubular scaffold along the graft; starting with the proximal to mid-proximal area which was closest to the inlet port of the bioreactor chamber. Eventually, the middle to distal

areas of the half sections is presented (Figure 45-52). The experimental culture parameters of study two are presented in Table 3.

Table 3: Summary of the cell confluency and cell sodding density for the statically cultured BVM in study two.

	Cell suspension	Surface Area	Cell Confluency	Sodding Density	Culture Time
Vessel 1	10×10^6 (cells)	$2\pi \cdot 15 \cdot 2.5 = 2.36 \text{ cm}^2$	60%	2.5×10^6 (cells/cm ²)	24 hours

2.3.2.1 *BBI Imaging*

2.3.2.1.1 *Vessel 1, 24 - HR at 2.5×10^6 (cells/cm²)*

Figure 45: The BVM shows increased cell coverage relative to the proximal regions from the vessels in study one.

Figure 46: Shows the BVM just next to the mid region, cell coverage stays fairly consistent between the two photos.

Figure 47: A mid-proximal view of the BVM. This photo shows the vessel region just right of the very proximal region.

Figure 48: Vessel 1 image of the right-distal region shows good cell coverage for being near the vessel middle edge.

Figure 49: Here the BVM is at the near proximal-mid region at 4x magnification. The most significant cell coverage was observed in this region.

Figure 50: Shows the BVM at a mid-distal range near the incision from the attached male barb ending.

Figure 51: An outer edge BBI shot of the BVM where minimal cell coverage was observed. Due to the bending of the scaffold, the outer edges appear unfocused.

Shown below (Figure 52) is an enlarged BBI image of the proximal-mid region of the BVM lumen. A 1 x 1 cm² grid was placed on the image to take the average cell count per cm². Since the length of the viewable portion of the BVM is around 1 cm, the number of images from the viewing area with 4x scope was limited to specific viewable regions away from the cut ends of the vessel. Cell average counts for the other vessel areas of study two can be located in Appendix F.

Figure 52: Vessel 1 towards the mid-distal region shows consistent cell adhesion near the vessel edge. The image shows the BVM at the proximal-mid region at 4x magnification with an average cell count of three random areas to make a better approximation. The most significant cell coverage was observed in this region with the vessel averaging $6-7 \times 10^6$ cells/cm².

2.3.2.2 *Histology*

The second half of the BVM was preserved in glutalderhyde until the appropriate time for histology staining. As oppose to the first BVM study, histological analysis on the BVM cross section occurred exactly one week after the BBI images were taken rather than a 2-3 week period.

The histology was carried out as described in Appendix D. Before the series of staining procedures, the vessels went through same preparation steps as in the first study. Once an adequate number of cut cross sections were prepared for the BVM, the sections were transferred onto glass slides via forceps. After twenty-four hours the slides were ready for staining and further processing.

2.3.2.2.1 Vessel 1, 24 - HR at 3.0×10^6 (cells/cm²)

Figure 53: The image taken was of the left proximal area of the cut cross section. The arrows point to the endothelial formation of HUVECs along the lumen lining. A consistent lining was present along this area of the vessel which was towards the proximal side of the bioreactor entrance port.

Figure 54: A bottom mid-proximal view of the vessel along the BVM cross section. No strong indications of endothelial formation along this portion of the cross section was found.

Figure 55: On the contrary, along the top region of the vessel cross sectional area of the BVM, there were stronger signs of endothelial development from the histology stains.

Figure 56: The BVM histology presented at a 10x view. Cell arrangement along the lumen was harder to distinguish among these cross sectional shots.

Figure 57: Cell grouping is less prominent compared to other stained sections of the proximal-mid region, as seen from the examples of scarce HUVECs within the ePTFE vessel.

Figure 58: The 10x view of the vessel shown here was a weaker development of HUVEC groups.

Figure 59: From the histology it appeared that cell numbers were less distinguishable in the right region of the vessel walls compared to the top-middle cross sections.

Figure 60: A zoomed view of the mid-top area of the cross section shows an easily distinguishable cell arrangement from the rest of the scaffold. Cell penetration was seen to some extent within the outer layer.

Figure 61: The endothelial lining from these images was more indicative of the stacked effect of the seeded HUVECs on the scaffold lumen.

Figure 62: This adjusted shot of the lining gives a better representation to the extent of the thickness of the ePTFE vessel. The endothelial linings along the intima layer alone represent only a fraction of the conduit thickness itself.

2.3.3 Results from Study Three

During study three, the four BVMs were constructed with cell confluency values that ranged from 60 - 80%. Similar to study two, the cell sodding density was estimated as cells/cm² from observing the final HUVEC confluency prior to cell sodding.

The P4 HUVECs in study three were cultured into P7 HUVECs to approximately 10×10^6 cells. A notable change was the amount of EC media used to resuspend each pellet prior to sodding from the previous studies. The culture flasks for vessel 1 and vessel 2 exhibited lower cell confluency around 70%. Vessels 3-4 were more confluent near 80%. Tables 4 highlights the cell density, flow, and take down points for vessels 1-4. A complete list of the cell sodding densities along with resuspension densities for study two and three are presented in Appendix K.

Table 4. BVM culture parameters for Study Three

	Cell suspension	Surface Area	Cell Confluency	Sodding Density	Culture Time	Flow Rate
Vessel 1	10×10^6 (cells)	$2\pi \cdot 15 \cdot 2.5 = 2.36 \text{ cm}^2$	70%	2.5×10^6 (cells/cm ²)	24 hours	10-15 RPM
Vessel 2	↓	↓	70%	3.0×10^6	24 hours	10-15 RPM
Vessel 3	↓	↓	80%	3.4×10^6	24 hours	15 RPM
Vessel 4	↓	↓	80%	3.4×10^6	24 hours	15 RPM

2.3.3.1 BBI Imaging

Following the same procedures of study two, the vessels in study three were preserved in glutalderhyde until the day of imaging. Imaging occurred two days after takedown and preservation of the four vessels. Below are the images of BVMS 1-4.

2.3.3.1.1 Vessel 1, 24 - HR at 2.5×10^6 (cells/cm²)

Figure 63: Shows vessel 1 at the distal end of inner wall with minimal cell coverage.

Figure 64: Vessel 1 at the proximal-mid region. The focused area of the image only shows a few viable cell adhesion points.

2.3.3.1.2 Vessel 2, 24 - HR at 3.0×10^6 (cells/cm²)

Figure 65: Any cell reflection is too difficult to distinguish and appears mostly bare; with minimal coverage across the prox-distal area.

Figure 66: A mid-distal shot leaves little to examine. Not much cell reflection from the surface was visible from the BBI photographs.

Figure 67: This image of the mid-distal area of vessel 2 follows along the line of the above images. Not much evidence can be drawn from magnitude of HUVEC adhesion success.

Figure 68: A few visible cells fluoresce in the proximal area of the vessel fragment.

2.3.3.1.3 Vessel 3, 24 - HR at 3.4×10^6 (cells/cm²)

Figure 69: The distal regions of vessel 3 with a few HUVECs spotted alongside the edges.

Figure 70: The middle region of vessel 3. Slightly more cell adhesion can be observed with these two images, however, the coverage remains too low to obtain any significant coverage count.

Figure 71: Unlike the above images, this view of the prox-mid region of the vessel can give a clearer count of cells.

Figure 72: Shows vessel 3 a little further along the vessel, there are slightly more focused and distinguishable cells in this capture.

Figure 73: This image is an expanded view of the prox-mid region; a cell average count is taken here to demonstrate the process of counting. Since the coverage is rather scattered than uniform, the cell count ranges in different areas but averages around 2×10^6 cells/cm² in this vessel shot.

2.3.3.1.4 Vessel 4, 24 - HR at 3.4×10^6 (cells/cm²)

Figure 74: The continuity of HUVEC presence across the ePTFE graft near the proximal region.

Figure 75: The BBI images from vessel 4 showed the most prominent and substantial cell adhesion, a view of the distal end of the BVM.

Figure 76: Similar to the BVM in study 2, the proximal end of the vessel shows cell stacking among the denser areas of HUVEC population.

Figure 77: A clearer shot of the prox-mid region shows the significant cell development formed on the lumen layer.

Figure 78: This expanded view shows a middle view of the prox-mid area of the BVM. Clearly, cell average counts become much easier to obtain within the focused area of the vessel.

Figure 79: Here the bottom area of the mid-distal region of the vessel is flattened out with a pair of forceps to assist in impeding the graft from curving upwards too much. A heavy cell population can be seen along the visible area of the ePTFE graft.

Figure 80: Here the average cell count was taken along 3 regions of the proximal-mid area of the BVM vessel. From the grid placement the average count of cells was between $6-7 \times 10^6$ cells/cm² across the presented area.

2.3.3.2 *Histology*

Assessment of cell adhesion with the scaffold was assessed through histology. The initial BBI preparation of the BVM cross sections occurred exactly seven days after capturing the BBI images. From the BBI images of study three it was expected that there would be signs of endothelial linings on the lumen of the scaffold. Similar to the earlier studies, the mid-distal half of the BVM was used for histological analysis, and was preserved in glutalderhyde during the week prior to examination.

The histology was carried out in a slightly modified manner compared to the protocol in Appendix D. A few changes were made with respect to exposures of the slides in certain staining reagents; the protocol is summarized in Appendix E. Before the series of staining procedures, the vessels went through the same preparation steps as described in the sampling preparation of the first two studies. One day from the initial preparation, the slides were ready for staining and further processing. Furthermore, a more significant subset of images was taken for vessel 3 and vessel 4 to help demonstrate a more typical BVM based on the initial BBI images, and cell density counts observed across the entire lumen section.

2.3.3.2.1 Vessel 1, 24 - HR at 2.5×10^6 (cells/cm²)

Figure 81: These snapshots of the cross sections of vessel 1 do not provide conclusive signs of endothelial presence.

Figure 82: Although a thin lining may be apparent from the first image, it is too hard to decipher the presence of cells from other staining reagents.

Figure 83: Vessel 1 at 10x view. Histology reagent is displacement around the lining formation is evident. Cells look present, but again are hard to clearly define from the stained reagent spots on the cross section.

Figure 84: Another angle of the right mid-proximal side of vessel 1 shows a lack of endothelial formation.

2.3.3.2.2 Vessel 2, 24 - HR at 3.0×10^6 (cells/cm²)

Figure 85: A right mid-proximal view of vessel 2 taken at 4x. A narrow endothelial lining is evident from the image.

Figure 86: Shows a top-mid shot of the BVM. A closer examination indicates a structured cell lining formed at a lower cell density.

Figure 87: Vessel 2 exhibits inconsistent cell lining development from top to bottom along the inner lumen.

Figure 88: As images were taken further up the vessel, more evidence of cell adhesion was observed.

Figure 89: HUVEC adhesion is minimal and but consistently lines the outer interstitial pattern of the ePTFE grafts.

Figure 90: Vessel 2 cross sections cultured at 10x. A thinner looking endothelial lining was present along the outer side of the BVM.

Figure 91: Substantial HUVEC grouping was present in fewer portions along the cross sectional views of vessel 2. Here the 10x image shows the red stained cell sections along the inner lining.

2.3.3.2.3 Vessel 3, 24 - HR at 3.4×10^6 (cells/cm²)

Figure 92: HUVEC lining seen along vessel 3. Here the 10x image shows the endothelium along the inner intima lining.

Figure 93: The circumferential images of vessel 3 demonstrate the consistency of the HUVEC lining with respect to the entire circular section of the graft.

Figure 94: Vessel 3 at a lower left view of the lumen cross section area at 4x. The extraneous bubbles were formed during the slide cover placement procedure

Figure 95: A continuation of the previous figure, where a moderately developed cell lining was observable across the bottom to right wall area of the scaffold lumen.

Figure 96: The cell confluency of HUVECs used in study three led to a more noticeable lining along the mid-proximal regions of vessel 3.

Figure 97: Easily distinguishable HUVECs have formed a consistent cell lining along the middle top luminal area of the vessel.

Figure 98: The noted HUVEC lining can be seen down in the entire right lumen section cultured at 24 hours.

Figure 99: In similar fashion the endothelial development of vessel 3 was observed to span the entire region along the lumen sides of the vessel cross-sections.

2.3.3.2.4 Vessel 4, 24 - HR at 3.4×10^6 (cells/cm²)

Figure 100: With denser cell seeding usages, vessel 4 showed more established cell lining networks along the lumen wall.

Figure 101: Vessel 4 is presented from top mid-view where the graft was noticeably folded during the staining procedure. An endothelial lining was observed down the lumen.

Figure 102: Vessel 4 is presented from the bottom mid-region view where the graft was folded during the staining procedure. A thinner endothelial structure was seen here.

Figure 103: From a mid-low angle view the endothelial remains consistently developed along the luminal lining.

Figure 104: A brightly lit 10x view of the bottom rim of the vessel shows nicely formed cell deposition.

Figure 105: The coverage was not confluent in all locations. Here the HUVECs appeared to penetrate into the graft interstices/pores. Cell stacking was evident at various levels of HUVEC adhesion.

Figure 106: Vessel 4 images just above the folded region of the graft cross section. HUVECs are slightly displaced from the inner lining.

Figure 107: A closer view of the left mid-proximal vessel wall shows the consistently developed endothelial lining along the lumen.

2.4 Conclusion of BVM Studies

2.4.1 General Observations of BVM Studies

Throughout each of the BVM studies observations were made that included any discrepancies that may have affected the end result of the endothelial lining confluency; for this reason and other details, the vessels were cultured with varied success between them.

With regards to addressing or improving the upkeep of the system, the experience from study one helped grasp possible situations that may occur at any point during a study in real time; such as bioreactor leakage, discontinued flow paths, or maintaining consistent flow regulation throughout the media reservoir tubing. The quicker turnaround time on the BBI imaging and histology in the latter studies proved to

be beneficial. Although the setups were performed in almost the same respective manner, there were still notable differences between each of the three studies with regards to cell adhesion consistency.

2.4.2 Study One Discussion

Each of the six cultured BVMs of study one showed minimal cross-sectional cell coverage from observations of the BBI images. The distal ends of the grafts showed even less evidence of coverage based on the lack of cell fluorescence on most of the viewable surfaces; these areas represented the ePTFE inner (lumen) surface. Also, it was not possible to approximate an average cell density value to the said scaffolds based on the ambiguity of the BBI images. However, based on the prior approximated cell seeding densities in Table 2 it was reasonable to predict that the increased concentration of HUVECs assisted the endothelial growth among vessels E and F.

Looking at the expected cell density values there are a few possible explanations for the lack of cell adhesion seen throughout each BVM in the BBI images. One factor may have to do with the two week time delay between graft enrichment in BBI solution, and when the fluorescent images were taken under the white light fluorescent microscope. Also, since BBI solution is mostly composed of highly deionized and purified milli-q water, it is possible that the DNA present among the cell population did dissolve or deteriorate to some degree based on the chemical properties of DNA. Furthermore, these conditions when combined, may have largely contributed to the voidance of HUVECs among BBI images.

In contrast, the histology images exhibited cells to an extent, but were still inconsistent in terms of lining cohesion around the scaffold circumference. The histology results for Vessels A-F showed signs of HUVEC adhesion along the inner lumen of the scaffolds. Although Vessels A and B could not confirm sustained endothelial development from the histology images, Vessels C-F had indications of cell adhesion and structured cell networks along the lumen layer. However, as noted before these findings also came with inconsistency, as a majority of the vessels had sectioned areas that possessed only short lengths of endothelial lining rather than uniform coverage, hence it was difficult to make reasonable assertions on specific parameters influence during the BVM trials.

Lack of cell presence or confluency in the cell cultures was also a considerable factor from one BVM to the next; this led to an undesirable variation in cell sodding density that was incorporated amongst the sets of vessels during the cell sodding procedure. Overall, cell adhesion was seen to some extent but the inconsistent endotheliums' of study one showed that closer examination of the BVM process was needed to attempt further reasoning behind the success or failures of future BVM setups.

2.4.3 Study Two Discussion

Expanding on the observations of study one, further examination of the effects of culture parameters on endothelial lining potential was carried out for study two with the hope of better understanding the complete BVM system. The characteristic differences

in the endothelium formations of the BVM constructs in the earlier study led to the specific questions of how cell densities, media flow adjustments, and volume changes of cultural media effects the end result of the endothelial lining.

The 24-hr culture period for the BVM in study two showed considerable increase in cell adhesion based on the BBI images. The post setup BBI imaging of study two occurred approximately 24 hours after the vessel was placed in 5 ml of glutalderhyde for preservation. The freshness of the BBI solution was also worth mentioning; a new batch of BBI was prepared relatively close to the imaging day. Moreover, the greatest areas of cell coverage were located towards the proximal-middle region of the BVM indicating the possible effects that gravity may have on cell migration within the vessel in correspondence with the flow rates of the culture media.

Histology results further reassured the presence of an endothelium along the luminal region of the BVM cross section. Most of the observable areas of the stained cross sections were covered with HUVEC deposition along the luminal lining. Despite the fact that the bioreactor chamber and media reservoir tubings/chamber were not gas sterilized in this study, there did not prove to be an adverse effect on the HUVEC adhesion or endothelium based on both imaging techniques. The relative success of cell adhesion and presence of a more consistent endothelial lining helped validate the installed protocol of the BVM setup, however the use of only static conditions allowed for further revaluation of applied steady flow rates on the culture system.

2.4.4 Study Three Discussion

Using information from developed BVMs on U-shaped ePTFE grafts by Dalton Chavez at the Cal Poly Tissue Engineering lab, certain procedural steps related to flow speed and ramp up in the U-shaped BVM development was compared with a variation of the implemented protocol from study two. The four BVMs in study three had varied results based on the BBI and histology images. BVMs 1 and 2 exhibited inconsistent cell coverage along the luminal lining. The BBI images for each vessel indicated some presence of cell adhesion but not enough to establish a consistent lining; as discovered from the histology images. It was worth noting that the bioreactor chambers for vessel 1 had leaks around the tubing connections. The top region of the ePTFE vessel was also exposed to air due to the drop in media. This occurrence may have not directly impacted the adhesion of HUVECs along the vessel, but may have imposed added stress that had some impact on the coverage of HUVECs.

BVMs 3 and 4 showed a substantial improvement regarding endothelial development and cell adhesion. These vessels were seeded at a slightly higher density than BVMs 1 and 2 and no complications occurred during their setups. However, the difference in cell seeding densities cannot completely explain why there was such a dramatic improvement with the overall endothelial coverage. Other factors may have included the overall effectiveness of the trypsin media to remove the cells from the T225 flask. A slightly higher volume and more vigorous shaking procedure of trypsin was introduced for the latter vessels based on the success or lack thereof from the previous

studies. This step may have increased the physical amount of cells that were introduced into each vessel.

2.4.5 *Future Outlook/Next Steps*

The major comparisons between the setups examined cell adhesion and possible factors that may have an impact on the HUVEC development in the current BVM system. Study two showed that a preconditioned ePTFE vessel at static conditions was able to sustain a minimal amount of HUVECs for 24 hours along the interior luminal lining. On the other hand, BVMs 1 and 2 of study three did not respond as well to the flow ramp up introduced during the transmural to luminal flow period. BVMs 3 and 4 in study three were vastly improved in cell coverage and cultured at a constant flow rate of 15 RPMs during the entire 24 hour culture period. Again, as discussed there are a number factors that may have contributed to the hindrance of cell adhesion for these vessels.

As the thesis progressed, it was necessary to initially expand on the implemented parameters from study one, and to look specifically at improving cell coverage on the lumen layer as well as consistent cell seeding densities for each vessel. Inconsistency of endothelial development across the ePTFE grafts indicates some flaw associated with the set-up or the assessment of the vessels and the setup processes involved with BVM setups.

It was reasonable to speculate a sudden introduction of high speed EC media was detrimental to the organization of HUVECs directly after seeding along the lumen

area of the scaffold. This in turn could be one cause of the drastically inconsistent results of study one. The BVM from study two as well as the latter BVMs from study three were successful at more consistent endothelial development with peristaltic flow rates around 10-15 RPMs. Nonetheless, the experimental parameters worth looking close at within the BVM setup such as flow-ramps, media usages, and cell population need more testing to examine the direct impact between these parameters and the growth of the endothelium.

After performing the preliminary BVM set-ups described in this chapter, it was still of emphasis to improve the methods involved with creating consistent BVMs. There were many experimental parameters that could have been further explored, such as flow rate, seeding density, media composition, etc. However, the thesis looked to bring a new approach to the tissue engineering lab with aims at improving sterility assurance throughout all facets of the lab. Moving forward, a quality control mindset was taken to analyze possible contamination routes that can potentially impact the BVM system and sequential processes, concepts such as sterility of materials, cell culture contamination, and evaluating cleanliness of the surrounding lab environment were closely scrutinized.

Chapter 3: Sterility Assurance of BVM Systems through Microbiological Practices

3.1 Introduction to Microbial Testing Significance and Microbiological Assessments

An undervalued component of TEVGs is the extreme delicacy that encompasses the growth, passage, and treatment of mammalian cells used in cell cultures. Whatever lineage the cell may be genetically, the conditions of the physical environment in which the cells are exposed to plays a huge role in either the success or hindrance of a scientific experiment. Having good sound knowledge in the field of sterility assurance and managing testing methods through microbiological analysis will be a beneficial tool to improve the BVM process within the TE lab. Therefore, the basic fundamentals on the growth of microbes will be explored along with the long-term significance of microbiological tracking in highly sensitive environments such as cell culture laboratories.

3.1.1 Early Forms of Microbiological Testing and Bacterium Growth

There are many pioneers to consider when looking back at the history of microbiology and early forms of testing practices. Many consider Louis Pasteur the “father” of microbiology. His ability to explain the concept of immunization and pasteurization has profoundly changed history. At the request of a distiller Pasteur was also able to demonstrate that alcohol fermentation is carried out by specific microorganisms (MOs) [76]. There were early scientists who came up with a plethora of

techniques that are taken for granted today, such as Robert Koch, who came up with Koch's postulates and demonstration of media use and sterilization, as well as the staining and sterilization of cells [76]. As more and more studies focused on MOs and their inherent characteristics, the concepts of bacteria growth and contamination risk became better understood.

3.1.2 Testing Bacteria Presence in Modern Era

When the science of microbial growth was better characterized, advancement in the areas of testing for microbes consequently followed. The temperature, available oxygen and nutrients, and competing bacteria in an environment are known as the most immediate extrinsic factors that affect the growth of bacteria [76, 77]. There are however attributes of MOs related to cell adaptability that were not well understood until the more recent modern era [77].

Traditional micro testing has outgrown many of the early techniques of sample collection and assessment in the last 20 years. While complex agars are still used for desired growth in controlled nutrients, selective agars that are used for the growth of select MOs with differential media are being phased out because of the inconvenience related with costs, time, material storage, and the emergence of microbial detection technology. While microbiology practices still rely on standard plate counts (SPC) for cultural growth to detect the presence of bacterial contamination in sensitive environments such as food process facilities, the shift towards rapid detection testing has become widespread [76]. Tools such as automated Polymerase Chain Reaction

(PCR), enzyme-linked immuno-sorbent assay (ELISA), gas chromatography (GC), and mass spectrometry are among the many methods used to discover the chemicals and living constituents present in food products, environmental samples, or in a various laboratory settings.

In addition to the evolving detection methods in today's science, the initiation of good laboratory practices (GLPs) has come a long way. Implementing sound regulated standards has become more common and expected among a variety of industries, including ones that test for the quality and chemical safety of consumer products. ISO accreditation of research and testing laboratories has established a scientific community that now sets a high standard of controlled and integrated systems in several fields of importance [78].

Expectations of the microbiological testing methods are to improve the sterility of the TE culture lab that is used for the creation of TEVGs. Through increased awareness of microbial contamination routes and knowledge of various bacteria testing methods used in many ISO approved testing facilities the prevention of cell culture contamination can be greatly reduced. Over time through microbial tracking feedback, the efficacy of the testing will be determined and adjusted as need be to create the most beneficial system of sterility assurance around the cell culture lab area.

3.1.3 Endothelial Cell Responses to Bacteria Exposures and Contamination

Endothelial exposures to various bacteria including, *E. coli* and *S. Aureus* have been well documented and is worth a closer look to help give some insight on some

possible outcomes of contaminations within a TE BVM. The capacity of these toxins to cause cell activation and mediator release in human endothelial cells was investigated and compared with the reaction of thrombin among such interfaces [79]. Hemolysin induced from *E.coli* and the alpha-toxin from staph was observed to be potent inducers of inflammatory and vasodilatory mediators in human endothelial cells; the elicited effects proceeded predominantly via activation of the phosphatidylinositol hydrolysis-related signal transduction pathway [79]. Furthermore, these toxins have shown to contribute to vasoregulatory and inflammatory disturbances and could potentially cause infection of an endothelium encountered in sepsis.

3.2 Cell Culture Contamination: Identification and Testing Methods

The biological contamination of mammalian cell cultures is alarmingly more common than one would imagine. In the mid-90's, statistics reported that between 11 – 15 percent of cultures in U.S. laboratories were infected with *Mycoplasma* species [80]. In 2010 a surprising 8.45 percent of cultures commercially tested from biopharmaceutical sources were contaminated with fungi and bacteria, including *mycoplasmas* [81]. In the following sections a variety of common bacteria groups and contamination sources will be examined to provide an overview of the remedial control methods of the initial microbiological testing routine introduced to the TE lab.

3.2.1 Contamination Sources

There are many basic preventative measures that are taken in various environments to reduce the risk of contamination, but contamination issues still occur.

Contamination may enter a laboratory or cell culture system through a variety of physical, biological, and chemical means. However, there is no substitute for practicing proper aseptic technique. Hence, understanding the routes of contamination as a practicing cell culture scientist is critical to the success of studies within a TE lab. The following passages will take a closer look at some of the more characterized routes through which cell culture laboratories may be contaminated

3.2.1.1 *Biological Sources*

Biological contaminants can be divided into two subgroups depending on the ease of detecting them in cell cultures. One group is easier to detect, which is considered most bacteria and fungi, while the more difficult to detect sources include *mycoplasmas*, viruses, and cross-contamination by other mammalian cells [78].

Contaminations can initiate a cascading series of metabolic events unique to each cell culture system and the contaminants [77]. There are multitudes of tests that can be run in order to confirm the identity and contamination status of each cell line.

In addition to direct bacterial intrusion, water that is routinely used in a cell culture lab can pose as a severe biological risk for contamination. Designated areas such as water pans under incubators, water baths, vortexes, and centrifuges are all subject to be splashed or contain water that can potentially lead to the microbial growth of aerobic MO's; in turn creating a cesspool for contamination risk. A common source of contamination is entry by a liquid "bridge" that forms when a droplet of culture medium remains between the culture vessel and its lid or on the neck of a culture medium bottle

[82]. There are however, more difficult to detect issues that are also introduced prior to the methods summary.

3.2.1.1.1 *Mycoplasmas*

Common biological contaminants such as *Mycoplasmas*, are a known threat within mammalian cell cultures. Mycoplasmas are the smallest (0.3 – 0.8 µm diameter) prokaryotes that reside in endosomes of mammalian cells [78]. Around 30 percent of all cell cultures are reportedly contaminated with mycoplasmas [83]. Mycoplasmas lack rigid cell walls, making them resistant to penicillin and related antibiotics. Since mycoplasmas lack a cell wall combined with their small size, they can sometimes slip through the pores of filter membranes used in sterilization [78]. In turn, attachment of mycoplasmas may cause host cells to leak and interfere with membrane-receptor function as well as signal transduction [77]. These problematic consequences of mycoplasma contamination have required in-depth research into various preventative measures of the species.

3.2.1.1.2 *Mycoplasma Contamination Testing with PCR methodology*

Since mycoplasma contamination is so prevalent in cell culture laboratories it is worth evaluating the significance of mycoplasma detection from specific methods. Although, the testing methods involve detection methodologies beyond the scope of introduced microbiological testing, the capabilities of these practices have relevance in the evolution of reliable testing capabilities-detections in a cell culture environment.

Polymerase chain reaction (PCR) testing methods will amplify a known sequence of DNA present in a sample and can relay various types of information depending on the desired analyses. To identify or distinguish a set of MOs or bacteria, PCR methods will generate a characteristic DNA sequencing of the sample which can be used to indicate the presence of a bacteria in a particular sample based on very sensitive logarithmic scale that quantifies DNA sequence detection [76].

Detection and elimination of mycoplasmas is time consuming and involves elaborate laboratory work. Treatment of mycoplasma in cell cultures was done experimentally through the incorporation of BM-Cyclin antibiotics being used in the research [84]. Some undesired effects of antibiotic contamination treatments in cell cultures were described, including; strong cytotoxicity leading to loss of culture, growth inhibition under treatment, loss of special cellular characteristics, and clonal selection of treated cells [77, 78, 85]. Among the obvious long-term detriments to a practicing cell culture laboratory, mycoplasma contamination is unfortunately a continuous downfall of many experiments that deal with reproduction and expansion of mammalian cell lines [78].

3.2.1.2 *Aerobic Microorganisms (MOs) and Coliform Groups*

Many industry standards have made aerobic plate count (APC) testing a mandatory procedure in facility inspection or water quality testing. APC levels in a given environment cannot indicate whether more harmful pathogens or viruses are present but can give an overall perspective on the cleanliness of tested areas or products, and

whether concern should be justified for further testing of specific microbes [76]. Some specific microbes that became a highly worrisome problem in the earlier 20th century included coliform contamination.

The term coliform has no taxonomic validity because the groupings are not based on genetics or biochemical reaction, but in general coliforms are aerobic, gram-negative, and non-sporeforming rods that ferment lactose; there are currently close to 20 or more species that may be classified as coliforms [76]. Within this chapter the main of focus is the detection of general coliforms; in addition includes the detection of *Escherichia Coli* (*E. coli*). Since the source of *E. coli* and other general coliforms would most likely come from a source outside the cell culture lab, the testing of this species allows an additional test for possible untreated contaminations of coliforms.

The *Escherichia Coli* species falls into the coliform species group that is classified under the name *enterobacteriaceae*; which categorizes fecal coliforms, *E.coli*, and other bacteria with the same family name. Escherich originally observed the ubiquity of the organism now known as *Escherichia coli* in human stools in 1887 [76]. Similar to the presence of total coliforms, the detection *E.coli* has served as a standard of sanitation and hygiene indication within processing facilities, water quality and food safety industries.

3.2.1.3 *Staphylococcus Aureus* (*S. Aureus*)

Another potential contaminant of concern in cell culture facilities is *Staphylococcus Aureus* (*S.aureus*). *S.aureus* bacteria is part of the normal bacterial flora found on human skin [76, 86]. The staph test is sometimes used with

corresponding TPC and coliform testing for a broader range of sterility feedback.

Accordingly microbial tracking of *S.aureus* in work environments has helped serve as an additional mode of sterility and hygiene indication [86]. Similar to the protocols of the aerobic and coliform bacteria plating, testing for *S.aureus* will take place throughout regions of the cell culture lab area. Confirmation of the specific staph species requires additional culture work; however, the petrifilm disk method for the detection of *S.aureus* provides a consistent barometer of species presence in desired testing points.

3.2.2 Chemical and Physical Sources

In one way biological contaminants are related to chemical and physical sources of contamination because the chemical contamination can be due to a biological-microbe influence on a physical or chemical entity within the lab. To better differentiate the two ideas, some common examples of physical and chemical contamination within a cell culture laboratory will be provided.

With respect to the physical sources of sterility risk, the role of CO₂ incubators plays a very profound role in maintaining the correct conditions for keeping cells viable, and thus is a liability to facility contamination. Mammalian cell cultures typically have settings most optimal for cell viability: a stable temperature of 37°C, a controlled pH around 7.4, with a controlled CO₂ level and a relative humidity of 95 percent [76-78]. The problem with these conditions is that it also provides an ideal environment for a range of biological contaminants that are considered normal flora (MO-bacteria) in and on human bodies [82].

In effect, when a researcher interacts with any part of the CO₂ incubator, flora from the skin or MO's on the clothing of an individual can transport a range of bacteria from outside the lab. Door handles, outer incubator, and chair surfaces are all common physical sources that harbor MO's from researchers' over time [78, 82]. Autoclaves, while intended to sterilize experimental materials can actually result in incomplete elimination of microbes if the device is overcrowded [78]. A cell culture can fail however based solely on the physical dimensions or design of the cell culture-ware and its influence on properties such as gas exchange, humidity, saturation density, and sterility [86]. A few examples of chemical risk exposures will be discussed.

Chemical contaminations are more severe and many times overlooked in cell culture practices. Culture media, bovine sera, reagents, residue on plasticware, or glass materials are the major sources of such cell culture contaminants of chemical nature [77, 78]. Unfortunately, many times chemical contaminations are not detected until the cell culture project is completed. This can obviously ruin the interpretation of results and the credibility of a research facility.

Contamination can come at any time of the media or serum lifetime. The raw materials used to make the base media as well as additional reagents, additives, and supplements can cause contamination if improperly stored or handled. It is vital that all chemicals/materials entering a cell culture system be of the highest purity, and preferably certified for cell cultures by a qualified vendor [87]. As mentioned previously, water baths, washing stations and other areas of a facility that house reagents used in a cell culture are susceptible to microbial contamination.

3.2.3 Consequences and Effects

While there are several types and forms of contamination, the effects from compromised cell cultures on the overall cell system can vary depending on the nature of the contamination exposure. Contaminations within a cell culture can alter the metabolism of the cell culture by producing metabolites and consume nutrients and essential amino acids [77]. Individual mycoplasma species are capable of fermenting sugars, oxidizing pyruvate, or metabolize urea and glutamine which can initiate a cascading series of metabolic events that may result in altered levels of protein, RNA and DNA synthesis [88]. Consequently, the regulation of cellular growth within a contained flask or culture system may be altered.

The regulation of cellular growth is based upon many characteristics including various enzymes, cytokines, and differentiated cellular functions. All these inherent traits of a particular cell line may be either induced or suppressed depending on the duration of the contamination and the actual alterations occurring in nucleic acid metabolism and chromosomal breakage [77]. Reiterating the severity of unnoticed contaminations, a few control and preventative measure techniques are discussed.

3.2.4 Control, Prevention, and Sterile Barrier Systems

Before utilizing any treatment or prevention techniques, the first approach should begin with determining the nature and extent of the existing contamination problem. Once the extent of the contamination is understood there are different treatments that can maintain desired cell function. For example, there are routine usage of antibiotics

for serial subcultures: including turning the cell culture system into a selective process for antibiotic-resistant bacteria and mycoplasmas [89]. So in order to be able to better assess cell culture treatment requires understanding the type of cells being dealt with and to remove any antibiotics from the system to accurately evaluate the incidence of bacterial or mycoplasmal contamination.

Prevention methods can be practical and if applied in a systematic methodology can prevent the compromise of sterile entities in the lab. This form of managing a cell culture laboratory is crucial for the successful creation of sterile barrier systems. The idea of sterile barrier systems comes in many forms of practice. Sterile pouches, laminar flow hoods, and even sterile gloves are a means to keep a controlled sterile environment regardless of laboratory size [78]. A look at basic techniques that minimize general bacteria growth within incubators and laboratory areas will showcase the highly sensitive nature of cell culture laboratories.

3.2.5 Assurance Techniques and Limits

With contamination routes and detection abilities reviewed, the microbiological testing methods being incorporated into the tissue engineering lab are a means to reduce the probability of a contamination occurring. The word “sterile” can be thought as an absolute term, but the confirmation that a product is sterile can only be defined in terms of a probability function [90]. Hence, a negative sterility test with no indication of microbial growth in samples of cell flasks, media, or the environment is not an absolute guarantee of sterility.

Contamination levels in media, media supplements, and sera can be reduced through preventative measures; such as by processing and storage. When it comes to media preparation, standard protocols should be put in place for the preparation of media and reagents to avoid common errors such incorporating the wrong component or amount of a particular ingredient. Other media preparation mishaps, such as the unintentional use of nonsterile supplies or solutions can be avoided with standardized procedures. If media is to be checked for sterility, large volumes of media should always be tested since a validated test may yield a false-negative as a result of random distribution of a contaminant [87].

An example of potential error in laboratory analysis of sterility is the testing of an end product or culture media that has been distributed to individual units such as bottles or cryovials. For example, if 5% of the bottles or cryovials are contaminated and 20 samples are tested, there is a 36.6% probability of getting a negative result [87]. The main emphasis being stressed is that one test of a single unit of a particular sample for any microorganism contamination is inadequate. Ideally, multiple samples should be tested and tracked over a period of time if contamination is detected at even the smallest of levels.

The sterile barrier systems used for related medical devices in a recent study demonstrated that microbial detection can be effectively examined at the sterility assurance level (SAL) of 10^{-6} . The SAL 10^{-6} level is currently required for sterilization procedures, i.e., a probability of not more than one viable MO in one million sterilized items of the final product [91]. Since MOs can be smaller than the pore size limits for

paper pouches, there was a need to evaluate the risk bacteria could penetrate the packaging slips. Microbial contamination issues with packaging depend on the environmental conditions such as storage conditions and transport, which may be associated with environments of high microbial load or meteorological pressure, and temperature changes [92].

In general the SAL level changes depending on the nature of a medical device product or tool, the inherent problem with the SAL level is evaluating the success of the proposed sterilization. At final inspection of a product it is nearly impossible since to evaluate contamination rates on the order of 10^{-6} since it cannot be quantitatively evaluated in experiments [90]. Since the aims of this chapter focus directly on improving the sterility of the laboratory area as a whole and not one single entity, such as a medical device, the application of SAL limits and studies aimed at suitable antimicrobial treatments can be used in conjunction with the sterile assurance techniques to formulate a sound testing design in eliminating specific microbes. Specifically, the goal of the work presented in this chapter was to implement methods to allow for the examination of microbial detection throughout the TE cell culture lab as well as the general research lab.

3.3 Testing Implementation

Before moving forward with the microbiological testing being presented to the tissue engineering lab, a summary of the expectations, goals, and desired feedback will be presented. To determine if there will be a direct correlation between the tracking of

microbes in the lab and the outcome of tissue engineering experiments is pretty limited and is more suited towards comparison testing among many studies over an extensive period of time. Rather, based on the research presented, the documented failures of cell cultures due to contamination from said examples will look to drive the methods portion of the study in hopes to control contamination for future cell culture studies based on the information involving the growth and tracking of MOs. Using methods formulated by accrediting bodies will also create a clearer and distinct set of protocols that will help the lab in the long term. Although the introduced styles of testing and prevention methods have been relatively consistent with microbial detection, there has been a shift towards accredited and approved testing regiments guided by larger regulatory agencies such as ISO, USDA, and HACCP.

3.3.1 ISO Testing Methods

Beyond the methods introduced in this chapter for microbial growth tracking, it is important to give a general summary of where some of these methods originated from and how they became an established rule in the modern era of science. The international organization of standards (ISO) body has played a large role in many industry practices to maintain and improve standardized processes and testing. The influence of ISO in microbiological testing has a broad spectrum, but is constantly shifting to meet today's difficult industry challenges. Examples of industries that utilize microbiological testing include the food safety industry, water quality testing, and cell culture facilities [76]. Most companies in these fields are shifting towards ISO

accreditation in all testing practices from the overwhelming push by the industry cliental requiring such certification.

3.3.2 AOAC, and HACCP Testing

A further expansion of ISO regulations specifically looking at microbiological methods, the Association of American Chemists (AOAC), Hazard Analysis and Critical Control Point (HACCP) systems, and USDA have shaped the principles and limits of chemical, physical, and microbiological limits with regards to testing consumer produce, processing facilities, and hazards within sensitive environments [93]. Below is a representative flow chart created by HACCP that summarizes the crucial control protocols applicable to a variety of quality control schematics.

Many of the methods incorporated in this chapter originate from models that begun with these institutional bodies. The role of the FDA is also worth mentioning as they have also set a standard of regulatory practices that is initiated today depending on the industry type, and the specific type of testing desired. The following section presents a summary of the methods for the types of microbes being analyzed and tracked around the tissue engineering cell culture lab.

Two of the major sources for these procedures come from the Compendium of Methods for the Microbiological Examination of Foods and 3M Microbiology. 3M testing kits included step-by-step instructional insert kits with most of the materials used in this chapter. A brief cost analysis comparison between the BVM setup and individual swab and sponge environmental sampling shows the cost effectiveness of implementing these practices to help save valuable resources and time. An average BVM setup cost including culture costs is nearly one hundred and ten dollars for one vessel [75]. With respect to the costs to do one swab or sponge sample with Petrifilm for the bacteria described in this chapter, using 3M materials costs only six dollars. In terms of required man hours the BVM setup from start to finish is at least a week long process that will require no less than 20 to 30 hours in the laboratory. Clearly this is a worthy justification to do sterility assurance checks of critical laboratory areas with the minimal amount of time required to do the environmental sampling. These simple processes can aid in preventing a contamination outbreak from ruining an entire setup.

3.4.1.1 *Swab Contact Method*

Lethen broth swab tubes were purchased from 3M microbiology for the swab sampling procedure. The method has been approved by the AOAC for use with disinfectants containing cationic surface active materials which make it convenient to swab areas that have been recently cleaned with such cleaning agents [93]. After all areas of the surface or object were swabbed, the swab was mixed in the letheen broth that was subsequently plated onto petrifilm which was incubated for further analysis.

3.4.1.2 *Sponge Contact Method*

Similar to the swab contact method the sponge sampling method used the same basic principles but allowed for the assessment of larger surface areas over a designated testing area. The target areas that were sampled were over a one square meter area using each side of the sponge to wipe the surface. After proper sample collection, plating procedures were performed based on the following methods.

3.4.1.3 *Aerobic Plate Count (APC)*

The commonly deployed aerobic plate count method is used as an indicator of aerobic bacterial population in a sample. These tests are based on the assumption that the cell will form a visible colony when mixed with appropriate nutrients in either an agar or today's rapid testing petrifilm test kits [76]. Also known as total plate coliform (TPC) or standard plate count (SPC), the test does not differentiate between the types of bacteria present and is only an indicator of bacteria that grow aerobically in mesophilic conditions [76].

Once the sample inoculum was distributed onto the gelatin surface, the petrifilm was transferred to a dry oven incubator at the conditions near 37°C without CO₂ - conditions. Standard growing incubators for petrifilm growth include 5% CO₂ and optimal humidifying conditions for bacteria; these conditions were not used for the culture durations of the inoculated petrifilms due to possible cell culture contamination risk with other TE studies. Nonetheless, relevant bacteria counts that were made will be presented and described in the results portion.

3.4.1.4 Coliform Count, *E.Coli*, and Staph Plate Count

The coliform count and *E.Coli* enumeration methods using the 3M petrifilm plating techniques followed nearly the same procedure as summarized in section 3.4.4. For this methods section 3M total coliform count and *E.coli* count petrifilm were used. This single petrifilm is able to detect both bacteria on the same petrifilm based on the family singularity of both bacteria groups [76].

Total coliform and *E. coli* colonies are observed to produce gas production from the presence of bubbles on the petrifilm surface. This is due to the fermentation of lactose by the coliform family. In addition, the identification of *E.coli* specifically entails gas production as well as the observation of the colony appearing blue. This chemical reaction is based upon the uptake of triphenyltetrazolium dye which produces the illumination [76]. Moreover, the remaining sections look specifically at the types of environments, and other significant areas in the culture lab that were tested either using swab and sponge contact methods.

3.4.2 Cleanliness Testing of Researchers Hands & Gloves

Cleanliness testing of hands and gloves was specifically introduced to track one of the most direct routes of contamination; the researchers hands. In general the researcher wants to maintain the most sterile interaction possible with any material that will directly contact cellular bodies and potential vascular conduits. While most cell culture practices take place with plastic gloves worn, this testing procedure compared the growth of the specified bacteria on hands with or without gloves used, as well as before and after cell culture procedures. Furthermore, the protocols described for this

specific type of testing in Appendix H will hopefully provide a long-term assessment of researchers' ability to keep the most sterility assured environment.

3.4.3 *Sterility Testing of Glassware, Equipment, and Media*

The sterility testing of all laboratory equipment can be done primarily through swab and sponge contact methods. This method also introduces general specifications that are mentioned in the compendium of methods for food microbiology for glassware and equipment safety. Concepts such as using hot water to wash equipment, pH testing of glassware or plasticware, and removal of “milkstone” and other residue deposits are some of the procedures that are looked into in further detail. In general the methods looked to add a line of security in aseptically sensitive environments such as cell culture and food testing laboratories. Further types of microbial evaluation methods are examined in Appendix H.

3.5 Results - Tracking

3.5.1 *Interpretation*

The following presentation of results is the culmination of two separate investigations into sterility assurance in and around the tissue engineering lab. Typical microbial colony counts for the petrifilm analysis were recorded as: cfu/ml for direct media or water sampling, cfu/50 sq cm for swab testing, and cfu/sq m for sponge analysis. Any discrepancies or mistakes in the protocol were recorded by the participants of this study and will be addressed in the discussion portion. All

participating researchers in this study were trained from microbiological testing models implemented in a food safety company that heavily utilizes these methods stemming from HACCP, AOAC, and USDA methods to test process lines, facility equipment, and multiple types of water sources.

3.5.2 APC Analyses, EC/TC Counts, Staph Counts

The initial sterility check study tested several key areas around the cell culture lab for any aerobic MOs that may have been present. Similar to the methods of the APC analysis, the EC/TC petrifilm was utilized to capture any potential growth of coliforms around the lab (Figures 109-110). The other type of microbe analyzed was *S. Aureus*. After an example demonstration of the swab and sponge contact method, the researchers performed the exercises individually. Some of the areas that were sampled included the laminar flow hood, cell culture counter surfaces, warm water bath, small and large culture incubators, and general lab areas. Subsequent follow-up studies were completed after the initial study; however Tables 5-6 document the initial and most recent study of recorded entries from petrifilm counts of APC, EC/TC, and *S. Aureus* over the designated areas.

Figure 109: No growth of *E. coli* or total coliforms was observed based on the lack of gas production. The white bubble areas on the grid represent air that was trapped underneath the petrifilm cover when plating occurred.

Figure 110: The APC petrifilm was utilized for the detection of aerobically growing MOs. Here a petrifilm reading of the cell culture lab counter shows bacteria growth to obtain the average bacteria count of the general lab side counter.

Table 5: Noted areas of microbial growth include the small CO₂ incubator as well the counter surfaces in the general lab area. However, other critical areas in the TE culture lab such as the flow hood or water bath showed no indication of microbial growth. Mixes of the sponge (cfu/sq m) and swab (cfu/50 sq cm) contact methods were initiated for the set of data.

	Aerobic MOs	Total Coliforms	<i>E.coli</i>	<i>S. Aureus</i>
209 Side Counter Clean	115 cfu/sq m	<1 cfu/sq m	< 1 cfu/sq m	< 1 cfu/sq m
209 Side Counter As Is	25 cfu/sq m	< 1 cfu/sq m	< 1 cfu/sq m	< 1 cfu/sq m
209a H ₂ O Bath	< 1 cfu/ml	N/A	N/A	N/A
209a CO ₂ Small Incubator	10 cfu/50 sq m	< 1 cfu/50 sq m	< 1 cfu/50 sq m	< 1 cfu/50 sq m
209a Flow Hood	<1 cfu/sq m	<1 cfu/sq m	<1 cfu/sq m	<1 cfu/sq m

Table 6: While a high TPC count for the general lab count was observed, the remaining test areas showed almost little to no growth. The presence of *S. aureus* on the side counter was a good indication of lingering issues that may be present in that given area.

	Aerobic MOs	Total Coliforms	<i>E.coli</i>	<i>S. Aureus</i>
209 Side Counter Clean	320 cfu/sq m	< 1 cfu/sq m	< 1 cfu/sq m	65 cfu/sq m
209 Side Counter 2 Clean	< 1 cfu/50 sq cm	< 1 cfu/50 sq cm	< 1 cfu/50 sq cm	< 1 cfu/50 sq cm
209a H ₂ O Bath	< 1 cfu/ml	< 1 cfu/ml	< 1 cfu/ml	< 1 cfu/ml
209a CO ₂ Large Incubator	11 cfu/sq m	< 1 cfu/sq m	< 1 cfu/sq m	< 1 cfu/sq m
209a Flow Hood	< 1 cfu/50 sq cm	< 1 cfu/50 sq cm	< 1 cfu/50 sq cm	< 1 cfu/50 sq cm

3.6 Discussion

The examination of the cell culture laboratory showed some signs of foreseeable problems that may come as a result of unclean surfaces. From the high counts of APC in the cell culture incubators to the general counters throughout the lab, a tangible improvement can be set for future studies. One well known contamination prevention method includes limiting the number of people sharing an incubator which will ultimately reduce the number of sources of contamination [82]. This goes hand in hand with the

idea that anytime an incubator door is opened by the user the risk of contamination increases. Since any one contaminant is liable to interfere with a tissue engineering experiment, the specification limits of any microbial count should center on the standard of keeping the lowest possible count of bacteria through the methods introduced; which is at less than 1 cfu/g.

Over the span of a few months two discrepancies were found throughout the protocols; including determining the average cell count on a petrifilm with high bacteria growth and the proper storage of an opened petrifilm package. The impact from these errors was hard to judge short term but would not have misconstrued the final counts of the first study since the petrifilm was brand new and no high bacteria counts were observed.

More testing and comparisons of microbial detection from un-cleaned to cleaned surfaces will assist in creating a protocol that will establish a more sterile cell culture environment. Any future laboratory specifications will have to be created around set limitations desired in the tissue engineering lab and what type of MO counts would be deemed acceptable or not. Discussions related to possible BVM testing incorporation is expanded on during the next chapter.

Finally, as the world of rapid detection technologies evolves so too will the various evaluations of detection design and versatility in cell culture systems. The current areas of TE experimental research will also take on a bigger impact in the medical world as more functionality of replacement vessels and organs progresses through modern science. Furthermore, the various sterility assurance parameters

introduced in this thesis are all worth giving closer scrutiny to as the lab continues forward.

Chapter 4: Discussion/Conclusion

4.1 Discussion of BVM Studies

The BVM studies presented in this thesis looked to expand on early research that presented a unique way to assess new and upcoming stents in a preclinical *in vitro* setting that could presumably evaluate endothelialization. There were unique obstacles that presented themselves within each study that required extra attention of the experimental parameters that were tracked during the BVM setup.

In the initial study, six BVM vessels were cultured over a one week period that evaluated intima layer development at time points of one, three, and seven days. Results were certainly mixed in terms of EC adhesion to the ePTFE scaffolds. With the first study each of the six vessels had a varying degree of HUVEC adhesion; from almost no cells present to slightly more cell adhesion based on the observed BBI and histology image references. Other factors to consider included setup experience, the incomplete removal of HUVECs from the culture flask, and subpar confluency of the HUVECs prior to cell sodding; these were some of the attributed discrepancies involved with the lack of cell density rates.

The latter studies presented built upon the first study with adjusted conditions of the BVMs to try and better understand the types of parameters of interest when reviewing the final results of TEVG constructs. The static flow conditions of the bioreactor system in study two showed that HUVECs were fairly spread on the lumen face of the ePTFE grafts. Thus, it was a fair question to ask whether or not the impact

of the steady flow conditions had an adverse effect on the HUVECs ability to maintain adhesion on the vessel lumen. In comparison the HUVEC confluency was also higher in this study and the procedures were more fluent in execution throughout the BVM process. Also, the single vessel setup did lower the risk of multiple setup errors, or flow pump issues that arose in dynamic and multi-vessel BVM studies. These are some of the observations that may have possibly contributed to the higher cell density value of the study two BVM with respect to the vessels of study one.

The final BVM study looked to expand on study two by developing four vessels with different flow parameters utilized by other researchers. Two of the four vessels were cultured at a constant 15 RPMs for 24 hours, while the other vessels were exposed to 10 RPM conditions for one hour under transmural flow and then increased to 15 RPMs for the remaining 23 hours under luminal flow. Notable problems with the setup were bioreactor leakages in the first two vessels setup under increasing flow, and a discontinuation of the media through the inlet port coming from the media reservoir. This may have resulted in the lack of consistent cell growth seen in vessels one and two of study three. The inconsistent flow application and even low cell confluency may have been additional factors as to why cell coverage was lower in the first two vessels. Vessels three and four were higher in cell density coverage, especially vessel four, were there no problems with leakage or flow, and the confluency of these cultural flasks were slightly higher than the flasks used for the first cell sodding procedure.

After careful observation of vessel four, which was the most confluent HUVEC lined vessel of all the BVM studies, it can be suggested that a constant flow rate did not

bare a negative effect on the cell adhesion process itself or lining development. However, the degree to which a steady flow system impacts HUVEC viability and morphology with regards to the imposed shear stresses under peristaltic conditions needs further classification. This is not to say that a very gradual increase in flow rate is detrimental to the BVM development, as was demonstrated in study one that showed increased flow rates on TEVGs had development of inconsistent endothelial linings [25]. However, a crucial observation was that more cell stacking and adhesion was observed in vessel four, a trait that can be investigated for further optimization in cellular consistency to utilize in conjunction with stent deployment in the current tissue engineering BVM model.

4.2 Progression of BVMs studies and Bioreactor Culture Systems

As the studies moved forward the key desired traits of the TEVGs seemingly improved as the studies progressed; yet there was still a gap within the results that could have been improved based on the individual processes of the BVM. Adding additional feedback and research into the current BVM protocol will continue to help future studies develop a culture process that consistently creates uniform endothelial layers on BVMs. Through the knowledge gained more accurate and precise assertions can be made as it relates to the impact that flow rates, cell densities, and cell confluency have on the overall BVM.

4.3 Discussion of Sterility Assurance Monitoring with BVM

Utilization

The sterility assurance portion of the work looked to pinpoint areas of the culture lab for microbial examination. The various species analyzed were aerobic MOs, generic *E. coli*, total coliforms and *S. Aureus*. Methods approved by AOAC and USDA bodies were used to capture and analyze the lab areas for possible target bacteria groups. After a couple of studies the research indicated that a low level of microbial growth was evident in and around the cultural lab. Although not surprising, the APC counts in the general lab area, as well as the incubators in the tissue engineering region indicate that there is room for improvement. Hence, further research can be introduced to incorporate possible testing during BVM trial studies on surfaces or equipment that is directly used in the cell culture process. Furthermore, sterility lab maintenance in between TE studies or at specific points in the BVM culture can indicate at when contamination may have occurred, and whether or not the sterilization procedures are being effective.

With the micro methods now implemented there are quality assurance checks that can be developed by the research group to create more flexibility in assessing the areas of the TE lab in conjunction with the BVM development. Since the focus of the tissue engineering lab remains to improve the direct methodologies involved with successful cultivation of vessels that can serve as intravascular device evaluators; a bi-weekly or monthly inspection of the facilities may be the most reasonable long-term incorporation of the microbiological methods. Moreover, this will assure that there are

no areas of the lab that are severely compromised, and hence potentially ruin TEVG setups in the future. It also provides a safeguard against any faulty aseptic techniques that may occur with people that have been newly introduced to TE lab.

While the general lab area outside the culture room is not exposed to tissue engineering construction, the concept of bacterial contamination from outside influences still raises concerns on what should serve as sufficient sterility control measures. If a sample returns a positive result for microbial growth several steps can then be formulated to best treat or assess the situation. Creating a quality control standard that develops a system where routine cleaning is performed and logged in is a simple yet effective type of control that can lead to modifications that best suits the TE lab. To do retesting of microbial presence in the same cell culture scenario with the same researcher may be a formidable avenue, as well as re-cleaning and testing in the area the researcher just completed culture work. The counter surfaces in the lab had a noticeable difference in colony counts between cleaned and un-cleaned surfaces which was expected initially. However, longer term tracking of microbial growth may demonstrate that further sanitation measures need to be implemented in the lab. Since the ideal laboratory surface would contain no indication of MOs through staged or random testing, this phase of the study can lead to further inquiries about developing the most optimal cleaning regiment while incorporating preventative measures of contamination routes to obtain consistently low bacteria counts.

Incorporating a consistent testing regiment the continued tracking of microbial growth can serve as a good indicator for the true cleanliness of the laboratory areas.

For example, the follow-up methods for tracking MO growth on a subject's hands or on bioreactor components presents limitless ways to test certain scenarios, but should be constructed in a way that best identifies the root cause of the contamination with respect to specific steps executed in BVM related protocols. Having to replace crucial bioreactor parts during a culture can be a detriment to keeping a sterile barrier system as described in the efforts targeting the validation of pulsatile perfusion bioreactors with HUVEC cultures [43]. Since there are dozens of individual steps within the BVM protocol and earlier parts of culture stages, there is a potential of many different testing points that can be established in conjunction with current TE studies.

Specifications can be set by the researchers accordingly; in highly sensitive areas such as the laminar flow hood and warm water bath there should be a low tolerance level, and would optimally be tested at multiple points during the cell passaging stages. If the initial testing shows an aerobic count of greater than one, then the researcher can perform the proper cleaning procedure and retest the area one day later, and test the area again during the final passing stage.

The amount of testing can be adjusted based on a consensus of acceptable average bacteria counts found over specific areas of the culture lab. Media testing, glassware, and plastic-ware testing are critical materials that also deserve more attention regarding contamination routes. These items can be carefully tested before and after being autoclaved as well as during the cultural duration of TEVG studies. Hopefully the research and methods described here can serve the TE lab in the

management of cell culture practices, and research that revolves around the sterile environment of the TE lab.

4.4 Future Goals

From the dozens of BVM related projects dealing with cellular development, scaffold construction, and bioreactor modeling; the TE lab has now started to transition to studying BVMs and their interaction with various stent constructs of newer generation models. Moreover, the success in repeatability of the BVM protocol within the TE lab at Cal Poly has allowed for the continued pursuit of improving modern metallic stent-intima assessments for 21st century medical devices.

An increasing trend in output demand of intravascular health devices will in turn require the continued sophistication of preclinical testing models to confirm safety standards. In essence, this need will continue to provide new goals and opportunities in TEVG research alongside the relentless pursuit of developing more realistic tissue models to evaluate medical device and drug delivery technologies. Improvements to the current BVM model through enhanced vessel mimicry by co-layer development, improved physiological conditions, and further tissue components found in blood vessels, are all realistic goals as the progression of blood vessel tissue engineering experiments moves forward.

Bibliography

1. Pullens, R.A., et al., *The influence of endothelial cells on the ECM composition of 3D engineered cardiovascular constructs*. J Tissue Eng Regen Med, 2009. **3**(1): p. 11-8.
2. Chan-Park, M.B., et al., *Biomimetic control of vascular smooth muscle cell morphology and phenotype for functional tissue-engineered small-diameter blood vessels*. J Biomed Mater Res A, 2009. **88**(4): p. 1104-21.
3. Lutz, S., *General Structure of Blood Vessels*. August 8th, 2009, Blue Histology: School of Anatomy and Human Biology - The University of Western Australia.
4. Cardinal, K.O., et al., *Tissue-engineered vascular grafts as in vitro blood vessel mimics for the evaluation of endothelialization of intravascular devices*. Tissue Eng, 2006. **12**(12): p. 3431-8.
5. Health, N.I.O., *Diagram of coronary angioplasty and stent placement - PTCA Stent NIH*. January 2006.
6. Cardinal, K.O. and S.K. Williams, *Assessment of the intimal response to a protein-modified stent in a tissue-engineered blood vessel mimic*. Tissue Eng Part A, 2009. **15**(12): p. 3869-76.
7. Liao, S., *Stem Cells and biomimetic materials strategies for tissue engineering*. Materials Science and Engineering C, 2008. **C28**: p. 1189-1202.
8. Thomas, A.C., G.R. Campbell, and J.H. Campbell, *Advances in vascular tissue engineering*. Cardiovasc Pathol, 2003. **12**(5): p. 271-6.
9. Bonnema, G.T., et al., *Assessment of blood vessel mimics with optical coherence tomography*. J Biomed Opt, 2007. **12**(2): p. 024018.
10. Girton, T.S., et al., *Mechanisms of stiffening and strengthening in media-equivalents fabricated using glycation*. J Biomech Eng, 2000. **122**(3): p. 216-23.
11. Salacinski, H.J., et al., *The mechanical behavior of vascular grafts: a review*. J Biomater Appl, 2001. **15**(3): p. 241-78.
12. Chamley-Campbell, J., G.R. Campbell, and R. Ross, *The smooth muscle cell in culture*. Physiol Rev, 1979. **59**(1): p. 1-61.

13. Owens, G.K., *Regulation of differentiation of vascular smooth muscle cells*. Physiol Rev, 1995. **75**(3): p. 487-517.
14. Li, S. and J.J. Henry, *Nonthrombogenic approaches to cardiovascular bioengineering*. Annu Rev Biomed Eng. **13**: p. 451-75.
15. Pankajakshan, D. and L.K. Krishnan, *Design of fibrin matrix composition to enhance endothelial cell growth and extracellular matrix deposition for in vitro tissue engineering*. Artif Organs, 2009. **33**(1): p. 16-25.
16. Lampugnani, M.G. and E. Dejana, *Interendothelial junctions: structure, signalling and functional roles*. Curr Opin Cell Biol, 1997. **9**(5): p. 674-82.
17. Isenberg, B.C., C. Williams, and R.T. Tranquillo, *Small-diameter artificial arteries engineered in vitro*. Circ Res, 2006. **98**(1): p. 25-35.
18. Webster, T.J., et al., *Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics*. J Biomed Mater Res, 2000. **51**(3): p. 475-83.
19. Wang, X., et al., *Sonication-induced gelation of silk fibroin for cell encapsulation*. Biomaterials, 2008. **29**(8): p. 1054-64.
20. Wang, X., et al., *Controlled release from multilayer silk biomaterial coatings to modulate vascular cell responses*. Biomaterials, 2008. **29**(7): p. 894-903.
21. Zhang, X., C.B. Baughman, and D.L. Kaplan, *In vitro evaluation of electrospun silk fibroin scaffolds for vascular cell growth*. Biomaterials, 2008. **29**(14): p. 2217-27.
22. Powell, H.M. and S.T. Boyce, *Engineered human skin fabricated using electrospun collagen-PCL blends: morphogenesis and mechanical properties*. Tissue Eng Part A, 2009. **15**(8): p. 2177-87.
23. Zhang, X., et al., *Dynamic culture conditions to generate silk-based tissue-engineered vascular grafts*. Biomaterials, 2009. **30**(19): p. 3213-23.
24. Pena, T., *PREPARATION AND CHARACTERIZATION OF ELECTROSPUN POLY(D,L-LACTIDE-CO-GLYCOLIDE) SCAFFOLDS FOR VASCULAR TISSUE ENGINEERING AND THE ADVANCEMENT OF AN IN VITRO BLOOD VESSEL MIMIC in Biomedical Engineering*. 2009, California Polytechnic State University: San Luis Obispo.

25. Inoguchi, H., et al., *The effect of gradually graded shear stress on the morphological integrity of a huvec-seeded compliant small-diameter vascular graft*. Biomaterials, 2007. **28**(3): p. 486-95.
26. Mitchell, S.L. and L.E. Niklason, *Requirements for growing tissue-engineered vascular grafts*. Cardiovasc Pathol, 2003. **12**(2): p. 59-64.
27. Yazdani, S.K., et al., *Smooth muscle cell seeding of decellularized scaffolds: the importance of bioreactor preconditioning to development of a more native architecture for tissue-engineered blood vessels*. Tissue Eng Part A, 2009. **15**(4): p. 827-40.
28. Kidd, K.R., et al., *Stimulated endothelial cell adhesion and angiogenesis with laminin-5 modification of expanded polytetrafluoroethylene*. Tissue Eng, 2005. **11**(9-10): p. 1379-91.
29. Di Luozzo, G., J. Bhargava, and R.J. Powell, *Vascular smooth muscle cell effect on endothelial cell endothelin-1 production*. J Vasc Surg, 2000. **31**(4): p. 781-9.
30. Yu, H., et al., *Dual cell seeding and the use of zymogen tissue plasminogen activator to improve cell retention on polytetrafluoroethylene grafts*. J Vasc Surg, 2001. **34**(2): p. 337-43.
31. Bala, K., K. Ambwani, and N.K. Gohil, *Effect of different mitogens and serum concentration on HUVEC morphology and characteristics: Implication on use of higher passage cells*. Tissue Cell.
32. Liu, Y., et al., *HUVEC cell affinity evaluation and integrin-mediated mechanism study on PHSRN-modified polymer*. Colloids Surf B Biointerfaces. **84**(1): p. 6-12.
33. Davis, J., *Isolation of Human Umbilical Vein Endothelial Cells (HUVEC)*. April 28th, 2007, Department of Molecular Biology and Biochemistry, University of California, Irvine.
34. Fujii, D.K., J. Cheng, and D. Gospodarowicz, *Phosphatidyl choline and the growth in serum-free medium of vascular endothelial and smooth muscle cells, and corneal endothelial cells*. J Cell Physiol, 1983. **114**(3): p. 267-78.
35. Hoshi, H. and W.L. McKeehan, *Brain- and liver cell-derived factors are required for growth of human endothelial cells in serum-free culture*. Proc Natl Acad Sci U S A, 1984. **81**(20): p. 6413-7.
36. Weiss, T.L., et al., *Serial subculture and relative transport of human endothelial cells in serum-free, defined conditions*. In Vitro Cell Dev Biol, 1990. **26**(8): p. 759-68.

37. Labitzke, R. and P. Friedl, *A serum-free medium formulation supporting growth of human umbilical cord vein endothelial cells in long-term cultivation*. Cytotechnology, 2001. **35**(2): p. 87-92.
38. Friedl, P.C., R., *An optimized culture medium for human vascular endothelial cells from umbilical cord veins*. Cytotechnology, 1989. **2**: p. 171-179.
39. Eckermann, C.W., et al., *Characterization and modulation of fibroblast/endothelial cell co-cultures for the in vitro preformation of 3-dimensional tubular networks*. Cell Biol Int.
40. Newman, A.C., et al., *The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation*. Mol Biol Cell. **22**(20): p. 3791-800.
41. Finkenzeller, G., et al., *Impaired in vivo vasculogenic potential of endothelial progenitor cells in comparison to human umbilical vein endothelial cells in a spheroid-based implantation model*. Cell Prolif, 2009. **42**(4): p. 498-505.
42. Luu, N.T., et al., *Responses of endothelial cells from different vessels to inflammatory cytokines and shear stress: evidence for the pliability of endothelial phenotype*. J Vasc Res. **47**(5): p. 451-61.
43. Chouinard, J.A., et al., *Design and validation of a pulsatile perfusion bioreactor for 3D high cell density cultures*. Biotechnol Bioeng, 2009. **104**(6): p. 1215-23.
44. Chiu, J.J., et al., *Shear stress inhibits adhesion molecule expression in vascular endothelial cells induced by coculture with smooth muscle cells*. Blood, 2003. **101**(7): p. 2667-74.
45. Bates, D.O., et al., *VEGF165b, an inhibitory splice variant of vascular endothelial growth factor, is down-regulated in renal cell carcinoma*. Cancer Res, 2002. **62**(14): p. 4123-31.
46. Konopatskaya, O., et al., *VEGF165b, an endogenous C-terminal splice variant of VEGF, inhibits retinal neovascularization in mice*. Mol Vis, 2006. **12**: p. 626-32.
47. Rennel, E.S., et al., *Recombinant human VEGF165b protein is an effective anti-cancer agent in mice*. Eur J Cancer, 2008. **44**(13): p. 1883-94.
48. Woolard, J., et al., *VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on*

- angiogenesis and endogenous protein expression. Cancer Res*, 2004. **64**(21): p. 7822-35.
49. Afkhami, F., Y. Durocher, and S. Prakash, *Investigation of antiangiogenic tumor therapy potential of microencapsulated HEK293 VEGF165b producing cells. J Biomed Biotechnol.* **2010**: p. 645610.
 50. Matsuda, T., *Recent progress of vascular graft engineering in Japan. Artif Organs*, 2004. **28**(1): p. 64-71.
 51. Miwa, H. and T. Matsuda, *An integrated approach to the design and engineering of hybrid arterial prostheses. J Vasc Surg*, 1994. **19**(4): p. 658-67.
 52. Diamond, S.L., S.G. Eskin, and L.V. McIntire, *Fluid flow stimulates tissue plasminogen activator secretion by cultured human endothelial cells. Science*, 1989. **243**(4897): p. 1483-5.
 53. Uematsu, M., et al., *Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. Am J Physiol*, 1995. **269**(6 Pt 1): p. C1371-8.
 54. Butler, L.M., et al., *Prolonged culture of endothelial cells and deposition of basement membrane modify the recruitment of neutrophils. Exp Cell Res*, 2005. **310**(1): p. 22-32.
 55. Orr, A.W., et al., *The subendothelial extracellular matrix modulates NF-kappaB activation by flow: a potential role in atherosclerosis. J Cell Biol*, 2005. **169**(1): p. 191-202.
 56. Huang, N., et al., *Hemocompatibility of titanium oxide films. Biomaterials*, 2003. **24**(13): p. 2177-87.
 57. Paschoal, A.L., et al., *Metallic biomaterials TiN-coated: corrosion analysis and biocompatibility. Artif Organs*, 2003. **27**(5): p. 461-4.
 58. Kastrati, A., et al., *Intracoronary stenting and angiographic results: strut thickness effect on restenosis outcome (ISAR-STEREO) trial. Circulation*, 2001. **103**(23): p. 2816-21.
 59. Simon, C., J.C. Palmaz, and E.A. Sprague, *Influence of topography on endothelialization of stents: clues for new designs. J Long Term Eff Med Implants*, 2000. **10**(1-2): p. 143-51.
 60. Yeh, H.I., et al., *Comparison of endothelial cells grown on different stent materials. J Biomed Mater Res A*, 2006. **76**(4): p. 835-41.
 61. Shimokawa, H., *Primary endothelial dysfunction: atherosclerosis. J Mol Cell Cardiol*, 1999. **31**(1): p. 23-37.

62. Yeh, H.I., et al., *Age-related alteration of gap junction distribution and connexin expression in rat aortic endothelium*. J Histochem Cytochem, 2000. **48**(10): p. 1377-89.
63. Yeh, H.I., et al., *Multiple connexin expression in regenerating arterial endothelial gap junctions*. Arterioscler Thromb Vasc Biol, 2000. **20**(7): p. 1753-62.
64. Yeh, H.I., et al., *Reduced expression of endothelial connexin37 and connexin40 in hyperlipidemic mice: recovery of connexin37 after 7-day simvastatin treatment*. Arterioscler Thromb Vasc Biol, 2003. **23**(8): p. 1391-7.
65. Lampin, M., et al., *Correlation between substratum roughness and wettability, cell adhesion, and cell migration*. J Biomed Mater Res, 1997. **36**(1): p. 99-108.
66. Yang, J., J. Bei, and S. Wang, *Enhanced cell affinity of poly (D,L-lactide) by combining plasma treatment with collagen anchorage*. Biomaterials, 2002. **23**(12): p. 2607-14.
67. Chung, T.W., et al., *Enhancement of the growth of human endothelial cells by surface roughness at nanometer scale*. Biomaterials, 2003. **24**(25): p. 4655-61.
68. Elbert, D.L. and J.A. Hubbell, *Reduction of fibrous adhesion formation by a copolymer possessing an affinity for anionic surfaces*. J Biomed Mater Res, 1998. **42**(1): p. 55-65.
69. Wang, W., et al., *A novel copolymer poly(lactide-co-beta-malic acid) with extended carboxyl arms offering better cell affinity and hemocompatibility for blood vessel engineering*. Tissue Eng Part A, 2009. **15**(1): p. 65-73.
70. Behravesh, E., K. Zygourakis, and A.G. Mikos, *Adhesion and migration of marrow-derived osteoblasts on injectable in situ crosslinkable poly(propylene fumarate-co-ethylene glycol)-based hydrogels with a covalently linked RGDS peptide*. J Biomed Mater Res A, 2003. **65**(2): p. 260-70.
71. Shieh, S.J. and J.P. Vacanti, *State-of-the-art tissue engineering: from tissue engineering to organ building*. Surgery, 2005. **137**(1): p. 1-7.
72. van Blitterswijk, C.A., *Tissue engineering*, ed. P.T. Clemens A. van Blitterswijk. 2008: Academic Press.

73. Hahn, M.S., et al., *Physiologic pulsatile flow bioreactor conditioning of poly(ethylene glycol)-based tissue engineered vascular grafts*. Ann Biomed Eng, 2007. **35**(2): p. 190-200.
74. Zhang, Z.-X., Chen, Bing, *Design of a novel bioreactor and application in vascular tissue engineering*. Applied Surface Science, 2008(255): p. 541-544.
75. Delgrammaticas, D., *DEVELOPMENT OF AN IN-VITRO TISSUE ENGINEERED BLOOD VESSEL MIMIC USING HUMAN LARGE VESSEL CELL SOURCES*, in *Biomedical Engineering*. 2009, California Polytechnic State University: San Luis Obispo.
76. *Compendium of Methods for the Microbiological Examination of Foods*. 4 ed, ed. F. Downs, Ito, Keith. 2001: American Public Health Association.
77. Lincoln, C., Gabridge, Michael, ed. *Cell Culture Contamination: Sources, Consequences, Prevention, and Elimination*. Animal Cell Culture Methods. Vol. 57. 1998, Academic Press.
78. Bates, M.K., Wernerspach, Douglas, *Cell Culture Contamination: Understanding and Managing the Risks*, in *Lab Manager: Technology and Operations*. May 2011.
79. Martinez-Lopez, F.M., Coburn J, *Responses of Human Endothelial Cells to Pathogenic and Non-Pathogenic Leptospira Species*. PLoS Negl Trop Dis 4(12): e918. doi:10.1371/journal.pntd.0000918. PLoS Negl Trop Dis 4(12): e918. **doi:10.1371/journal**.
80. Ryan, J., *Understanding and Managing Cell Culture Contamination*. Corning Life Sciences, Technical Literature, 2008.
81. Armstrong, S.E., J.A. Mariano and D.J. Lundin, *The Scope of Mycoplasma Contamination within the Biopharmaceutical Industry*. Biologicals, 2010. **38**: p. 211.
82. Bates, M.K., Wernerspach, Douglas, *Cell Culture Contamination Part 2: The Role of the CO₂ Incubator in Reducing Contamination*, in *Lab Manager: Technology and Operations*. June 2011.
83. Uphoff CC, D.H., *Detection of mycoplasma in leukemia-lymphoma cell lines using polymerase chain reaction*. Leukemia, 2002. **16**: p. 289-93.
84. Jung, H., Wang, Hsin-Shih, *Detection and Treatment of Mycoplasma Contamination in Cultured Cells*. Chang Gung Med, 2003. **26**(4).

85. Uphoff CC, D.H., *Elimination of mycoplasma from leukemia-lymphoma cell lines using antibiotics*. Leukemia, 2002. **16**: p. 284-8.
86. Adams, R.L.P., *Cell Culture for Biochemists*. Elsevier, New York, 1980.
87. Johnson, R.W., *Quality assurance of tissue culture media used in the biotechnology industry*. BioPharm, 1990. **3**(2): p. 40-44.
88. Miles, R.J., *Cell Nutrition and Growth in Mycoplasmas: Molecular Biology and Pathogenesis*. American Society for Microbiology, 1992: p. 23-40.
89. Roberts, M.C., *Antibiotic Resistance: Mycoplasmas: Molecular Biology and Pathogenesis*. American Society for Microbiology, 1992: p. 513-523.
90. Von Woedtke T, K.A., *The Limits of Sterility Assurance*. GMS Krankenhaushyg Interdiszip, 2008. **3**(3).
91. *European Commission (EC) Guidelines of Good Manufacturing Practice for Medical Products*, P. Industry, Editor. 1990. p. 853-74.
92. T., D., *Comparison of State-of-the-Art Technologies for Aseptic Processing*. Medical Device Technology/BioPharm Europe, 1999. **11**: p. 52-57.
93. Pierson, M.D., Corlett, Donald A., ed. *HACCP Principles and Applications*. 1992, Van Nostrand Reinhold.

Appendices

Appendix A – Study One BVM Setup - Fall '09: December, 2009

PART I: Culture Process

Thawing HUVECs

The necessary experimental materials needed for the thawing stage included: one sterile T75 flask, one bottle of HUVEC media, and one vial of unfrozen HUVECs. In order to retrieve the frozen HUVEC vial a pair of thick custom woven mitten gloves were used to remove the vial from the N₂ tank. After retrieving the cells, the HUVECs were thawed by placing the small vial inside a Shel Lab warm water bath at 37°C. It was critical not to submerge the HUVEC vial completely in the water bath to avoid contamination through water submersion around the vial cap as well as from possible aerobic microorganisms in the water.

Later a T75 flask was brought under the flow hood where 20 ml of EC media was slowly pipetted into the T75 flask. The HUVECs were transferred from the cryovial into a T75 culture flask. Finally, as with the conclusion of every cell culture stage, the hood surface was cleaned with 70 percent IPA and all material waste was disposed of accordingly into biohazard containment.

Passing Stages

Next, the previously prepared and thawed HUVECs were removed from the incubator. After recording the confluency the flask was brought underneath the hood.

When the confluency was recorded, the media solution was aspirated out using a vacuum operated pump and an aspirating pipette. Afterwards, 2 ml of (DCF-PBS) was added to the flask of the harvested HUVECs. To dislodge any clumped or scattered areas of HUVECs on the flask surface, 3 ml of trypsin was pipetted into the T75. Prior to the addition of trypsin, the media was put on a vortex to ensure that vital proteins were thoroughly mixed prior to its introduction to the HUVEC population.

At this stage the flask was observed for dislodged HUVECs floating throughout the trypsin solution. When the cells appeared to be in a free suspended state, 7 ml of EC media was pipetted into the flask to counteract the effect of the trypsin media. The amount chosen was slightly arbitrary; however, at a minimum, the equivalent amount of EC media to trypsin was required to be added to deactivate the trypsin. A prolonged exposure to trypsin has shown detrimental signs to cell walls and vitality rate [42]. To recap, the HUVECs were suspended inside 10 ml of total cell media (7 ml EC media + 3 ml trypsin); the cell solution was then thoroughly mixed and shaken with a pipette aid for proper inoculation of media. After adequate mixing, the entire amount of cell solution was transferred into a prepared T75 with fresh media.

However, during the procedure the HUVECs were mistakenly transferred to only one separate T75 flask instead of two. Moreover, the 1st passing stage failed to expand the volume or population of HUVECs as intended, but instead only served as a media replacement for the HUVECs already housed in the T75 flask. In other words this stage did not improve HUVEC confluency in means of population doubling; a characteristic that was confirmed through the white light microscope.

To increase the HUVEC population, a population doubling to a P5 was performed the following day as the HUVECs were transferred into a new T75. The goal of this passing stage was to perform a 1:6 pass by properly transferring the HUVECs inside the T75 into two T225 flasks. Upon observation after the first passage, the HUVECs were recorded at 100 percent confluency.

Later, standard cell culture techniques were performed on the HUVECs within the T75s. The crucial step was ensuring proper removal of HUVECs from the flask surface with the incorporation of trypsin solution in an adequate amount of time. During this passing trial EC media was used to deactivate the trypsin. To capture as many HUVECs as possible from the flask, the cell media solution was mixed and shaken with a pipette aid across the flask.

Following the thorough mixing, the cell solution was pipetted and transferred into T225 flask with fresh EC media. An equal amount of HUVEC solution was pipetted into each prepared T225 flask. The HUVECs were successfully passed from a P4 to P5 and as they were returned to the Shel Lab incubator.

The final stage of passing for the initial BVM setup passed the HUVECs from a P5 to a P6. For a HUVEC population at P6 the expected outcome was to obtain a cell population of 1×10^6 cells/cm², although this amount may have been lower than expected because an extra day was present in between passing stages of the 1:6 pass. Confluency of both flasks was recorded at 100% just prior to passage. As before, similar cell culture techniques were performed on each T225 containing HUVECs for this passing point. Now the T225 was shaken and observed for HUVECs floating

around in the trypsin solution. Finally, the HUVEC media solution from each T225 flask was pipetted and evenly distributed into two separate T225s. The HUVECs were successfully passed into four new T225 flasks at a P6 population.

Feeding Cells

Maintaining the desired environment for HUVECs during each culture stage was a key ingredient to acquiring a healthy and confluent population of cells. Although the EC media provides the proper nutrients and chemical environment to preserve the integrity of the HUVECs, it was required that the EC media be replaced between passing stages to maintain the adequate nourishment needed to keep the HUVECs in exceptional health. With each flask devoid of EC media with the aid of a vacuum pump, fresh EC media was pipetted into each T225 respectfully.

Preparation and Preconditioning of ePTFE Grafts

The denucleating process helps remove trapped air from the interstices of the ePTFE grafts. 10 ml vials of 70%, 100% ethanols were prepared, along with six 10 ml vials containing CM that was properly degassed in the appropriate flask. Soaking scaffolds in CM helped with protein adsorption, which improved cell adhesion of HUVECs to the ePTFE scaffold during the BVM development.

Materials and Sterilization

In order to correctly configure the BVM setup all bioreactor materials, scaffolds, and tubing, was either steam sterilized or passed through an ethylene oxide (EtO) gas sterilization. Other accessories that were autoclaved included forceps and cutting scissors as well as a degassing media flask.

Materials included six separate biochambers, accompanying tubing and valves, and with modified fittings assembled inside the bioreactor to create the appropriate spacing for the ePTFE graft (Figure 16-18). A separate 50 ml media conical that served as the media reservoir chamber was packaged into sets of two. All materials were dropped off at the Vet Clinic one week prior to preparing and condition the ePTFE grafts.

To prepare the culture media used in the BVM experiment, crucial growth factors and chemical agents were mixed and filtered into a sterile media bottle. All steps had to be performed underneath the flow hood and carefully executed using aseptic technique. Two bottles of BR media and one bottle of EC media was needed for the six BVM setup. All media bottles were previously autoclaved prior to preparation.

PART II: BVM Protocol: Vessel A-F Preparation

1. Wash hands, spray down gloved hands with IPA, spray down the hood, and maintain aseptic area.
2. Retrieve Bioreactor media and conditioning media, spray down with IPA, and bring into hood.
3. Bring in sterilized packaged bioreactor chamber under hood. Pull out chamber from package by tugging on outside valve (Do not ever run hands over sterile chamber body once opened).
4. Fill chamber with bioreactor media, go just over the fitting level.

5. Bring in sterilized packaged ePTFE grafts into hood. Open package carefully and dump graft into bioreactor chamber (1 graft per chamber). Do not let graft fall onto hood surface or nonsterile area.
6. Obtain sterile-sterile gloves from cabinet. Insert right hand first carefully, use left hand to navigate the non-sterile bottom portion of the glove over right hand. Then carefully use your gloved right hand to navigate left hand properly into glove. Avoid any contact w/ nonsterile surfaces during this process!
7. Insert fingers into chamber and secure grafts into fittings. Twist the barbed fittings onto the female ends of the grafts, not the other way around.
8. Dispose of sterile-sterile gloves and put on regular latex gloves. Obtain sterilized packaged bioreactor chamber lids and open under sterile hood; again avoid contacting inner surface of the lid, instead use the lid flaps to grip the lid and navigate it onto the bioreactor chamber, snap the lid closed.
9. Prepare reservoir chambers; obtain packaged reservoir tubing/pump tubing along with clean 50 ml media vials (1 per package). Open tubing package under hood. Assemble accordingly to optimize distance of pump tubing relative to peristaltic pump (see diagram). *Brown pump tubing should be attached to the inlet port side of the media vial cap.
10. Fill media vial w/ 40 ml of conditioning media (CM).
11. Allow CM to prime the tubing by opening the valves on the tubing and turning the media vial upside down.
12. When it is seen that CM has flowed all the way back to the media vial, clamp off both ends of tubing. Set aside media vials w/ properly attached tubing until the conditioning stage.
13. Now it's time to prime the attached ePTFE graft with CM.

14. Obtain two troughs per ePTFE graft being primed (per bioreactor chamber), one for catching the fluid from the exit port, the other trough is used to hold the CM at the entrance port as you suction it up with a syringe pump.
15. Fill “inlet” trough with 6-7 ml of CM, and use syringe pump to suck up all the media.
16. Before injecting CM with syringe pump, it is crucial to have valve orientations opened in the following order: inlet stopcock (white) pointed away /opposite (left) from fluid flow, exit valve open (turned parallel to tubing), and lumen valve open/transmural valve closed.
17. Insert syringe into inlet port and start slowly applying constant pressure on syringe; when media in syringe reaches 1-2 ml, close lumen valve to allow flow transmurally, and apply pressure to push remaining fluid transmurally through graft. STOP pushing on the syringe right before the last bit of media is pushed through to avoid pushing air through the tubing and into the ePTFE graft pores.
18. Now close off transmural valve, exit valve, and inlet valve to avoid spillage (in preparation for the next step).
19. Now the assembled bioreactor with the primed ePTFE graft is ready for its first conditioning pump run.
20. Bring in peristaltic pump beforehand into the large incubator; spray down with IPA before doing so.
21. Transfer assembled bioreactor from underneath the hood and into the large incubator as well as the media reservoir assembly.
22. Assemble bioreactor setup with media reservoir and tubing appropriately onto the peristaltic pump. **Make sure gray tubing part is fitted into the pump trays correctly. This tubing should be attached to so that small intermediate tubing attaches to the inlet port of the bioreactor chamber.

23. Now, with setup complete, open lumen and exit valve and make sure inlet stopcock is oriented upwards so as to flow through the tubing. (Transmural valve should be closed initially).
24. Turn on peristaltic pump and then push start button. Run pump at high speeds (150 RPMs) for 10 minutes for luminal conditioning. Make sure you see a drip in the media chamber. After 10 minutes of pumping, stop pump.
25. Now allow transmural flow conditioning (by closing off lumen valve). Start pump again w/ transmural flow and run pump at slightly lower speeds for 5 minutes (70-100 RPMs). 70 rpms was used as operating speed.
26. Stop pump, close off all valves, clamp off media reservoir tubing to avoid dripping media everywhere during the transfer stage of the bioreactor setup back underneath the hood.
27. Now ready for cell sodding stage.

PART III: Cell Sodding and Incubation

28. Obtain your culture flasks (T225s) from small incubator, check cell confluency prior to sodding.

Observations:

- **Vessel A: 60 - 70%**
- **Vessel B: 60 - 70%**
- **Vessel C: 80%**
- **Vessel D: 80%**
- **Vessel E: 100%**
- **Vessel F: 100%**

29. Use standard cell culture techniques to isolate cells to eventually get a pellet of HUVECs.
30. First, aspirate out media from T225 flask, rinse w/ DCF-PBS, aspirate out again, then add 6 ml of trypsin-EDTA (this amount can vary) to flask. Then add 7 ml of CM (or equal-greater than amount of trypsin used) to detrypsinize. (Use BR media if CM is low). Pipette solution up and down a few times to make sure most of the cells are captured. Beat flask with palm of hand for 3-5 minutes to further release cells (Check cells again under microscope to reaffirm presence of cells in media and that the trypsin worked).
31. Pipette media from flask into a 50 ml media vial/conical.
32. Place conical inside centrifuge machine.
33. Balance conical w/ another conical (could be cell media conical or just plain water).
34. Run centrifuge for approximately 4 minutes on setting 4.
35. Once complete, take conical out slowly to avoid dispersing pellet back into solution, and bring conical into hood!
36. Now aspirate fluid from vial carefully, making sure not to suck up the pellet!
37. Now resuspend pellet with 5 ml of HUVEC/EC media per bioreactor chamber. If you plan on sodding multiple grafts with a single pellet (for example if you want to sod 2 grafts, resuspend pellet with 10 ml of HUVEC media, and evenly divide the HUVEC cell solution per chamber/5 ml per graft).
38. With 10 ml pipette suck up all of the media with dispersed pellet, make sure the entire pellet has been resuspended.
39. Now obtain two troughs again per bioreactor, add the crucial sucked up cell solution into the inlet trough located in front of the inlet port.

40. Try and maintain sterility here; avoid running hands over trough once cell media has been dispersed.
41. Now with 4-5 ml of EC media in trough, the vessels are ready to sod. Once again make the valves are oriented are correctly.
42. Lumen valve should be closed!! Exit valve should be open and be set on top of the exit trough. Inlet port should be pointed away from graft (left) so that cells flow into ePTFE graft.
43. Using a clean syringe, suck all media, avoid sucking up air.
44. Attach syringe to inlet port, slowly apply pressure: stop applying pressure near the end to avoid pushing air bubbles through the ePTFE graft pores!
45. Once the graft has been sodded, “chase” the HUVEC cell solution injected by injecting 3 ml of HUVEC media into graft to ensure that the HUVECs were properly placed inside the interstices of the ePTE grafts.
46. Now ready to transfer bioreactor setup back into large incubator and connect to pump and media reservoir (media reservoir should be switched out so it contains fresh HUVEC media instead of the previous CM media!).
47. Close off exit valve and inlet stopcock to avoid spillage of media during transfer.
48. Reassemble bioreactor system: bioreactor chamber to media reservoir and media reservoir tubing into pump and back into the inlet port.
49. Now start pump at very low speeds: Maintain 14 rpm transmural valve flow for 1/ 2 hr.
50. Once a consistent circular flow has been established close incubator door and turn on CO₂ tank; this is not necessary for 24hr cultures).

51. After 30 minutes, come back and open lumen valve (thus disallowing transmural flow).
52. Gradually ramp up flow speeds accordingly over course of one, three, and seven days.
53. Repeat process for other vessel(s) being sodded.
54. Come back on scheduled predetermined days to ram up flow (if necessary) accordingly and eventually take down/fix vessels.

Fixing/Flow-Ramp Up

The following sections describe the stepwise procedure involved with the steady flow ramp-up of the BVM vessels for the setups described in study one.

Fixing BVMs and Flow Ramp-Up One

After 24hr two of the six BVMs were taken down from the bioreactor culture conditions and stored in 12 ml of histochoice until further examination, while the peristaltic pump speed was increased for the remaining vessels. Flow was ramped up to 35 RPMs for the four remaining BVMs at this stage. After two hours the flow was increased to 70 RPMs. Finally, two and half hours later, the flow was increased to 120 RPMs.

Also, it was preferred that EC media be used inside the media reservoir chamber instead of CM during the BVM process. To compensate for this change in the BVM protocol, the CM for the four remaining media reservoirs was replaced with four conical vials containing 40 ml of EC media. This required a stoppage in flow and an absence of

applied shear stress on the HUVECs. The remaining vessels were cultured with flowing EC media for the duration of study one.

To remove the BVMs from the bioreactor chamber a clean razor was used to cut the vessels from the barbed fittings and sutures. Bioreactor media was disposed of into a hazard waste bag as well as any remaining CM inside the reservoir chamber; also any non-disposable equipment was cleaned and flushed with hot water. Finally the vessels were put inside a small vial containing histochoice. The vial was labeled with the time of take down and location of the distal and proximal ends of the vessel with respect to its position inside the bioreactor chamber.

Fixing Two BVMs and Final Flow Ramp-Up

At the 72 hour mark, two more BVMs were fixed and preserved in histochoice. After the BVMs were removed from the peristaltic pump system, the latter two BVMs were exposed to increased pump speeds of 200 RPMs for the remainder of the BVM culture.

Fixing Final Two BVMs

One week after the initial BVM setup, the final two vessels were taken down and fixed in histochoice for examination. The peristaltic pump was taken down and cleaned down with IPA as well as the CO₂ incubator shelves. Other materials and media were disposed of accordingly. The six BVMs were now ready for further analysis through BBI and histological examination to assess the cellular growth and adhesion along the lumen or interior walls of the grafts.

Appendix B - Study Two BVM Setup - Summer '10: June, 2010

PART I: Culture Process

Thawing HUVECs

In study two, cryopreserved P8 HUVECs were used as the initial cell passing point. Furthermore, the HUVECs were transferred into the T75 and put back into the Shel Lab incubator until the first passage was performed.

Passing Stages

Moving forward in standard fashion, all steps carried out were performed in sterile aseptic technique. The cell passage steps in this procedure were performed in identical manner to study one (see Appendix A passing stages).

Notable differences between study two and study one came from the amount of trypsin and HUVEC media applied. More trypsin was used this time around to ensure that the entire T75 was adequately covered to help dislodge as of much of the HUVECs as possible. Instead of the 2-3 ml, 6 ml of trypsin was used in study two. Initially the first study of HUVEC passages into T75s used 20 ml of prewarmed (EC) HUVEC media per flask, however, all precoating stages for study two and study three consistently uses 12 ml of prewarmed HUVEC media for T75 flasks and 36 ml for T225 flasks. Now, a T225 with P9 HUVECS was prepared and returned back into the incubator were they were subsequently fed with fresh HUVEC media after 48 hours, and then ready for cell sodding on setup day.

Feed Cells

Cell media replacement was only done 1x during study two. After 24 hours of incubation following passing stage 1, the HUVEC media within the T225 was replaced with 36 ml of fresh prewarmed HUVEC media. The HUVECs coated on the surface of the flask were now replenished with fresh nutrients and growth factors provided by the HUVEC media. Finally, the T225 was returned to the incubator in preparation for ePTFE denucleation.

Preparation of ePTFE Grafts

ePTFE grafts, 3.5 cm long and 2 mm in diameter were used as the BVM scaffold structures for study two from Bard incorporation. Similar to study one, the graft material was mounted onto barbed fittings and then sutured around the fitting for proper closure and insulation. Once the graft was prepped, it was inserted into an autoclave package with an indicator strip and autoclaved for 30 min at 250° F with an additional 30 minutes for drying inside the Ritter M9 Autoclave. The graft was later retrieved before implantation inside the BVM model where they were denucleated and conditioned in a series of treatments in graded ethanols and CM.

After the autoclave process was complete, the ePTFE graft was ready for denucleation. As in the previous study for the ePTFE scaffold, corresponding 10 ml vials were filled in advance with 70 percent ethanol, 100 percent ethanol, and CM.

The ePTFE graft transfer procedure strictly followed the steps of study one. An important modification on the priming procedure from study one to study two came at

the final step; instead of leaving the vessel under the flow hood, the scaffold was transferred into the incubator to remain at more physiological conditions until setup day.

Materials and Sterilization

Setup materials in study two were not gas sterilized based on the minimized culture time involved. Yet, aseptic sterile techniques were still maintained throughout all phases of the experiment. The materials listed in table 5 represent the materials involved with the study two and three systems. It is worth noting that any media bottle used in the project was autoclaved individually prior to media preparation. Autoclaving any used material or accessories/tools that directly or indirectly was involved with the development of the HUVEC culture was a constant necessity in the BVM setups to maintain an aseptic-sterile environment.

The bioreactor (BR), HUVEC, and Conditioning Media (CM) used in study two and three contain the same components as mentioned in study one. So forth, the preparation in making the medias also followed the same procedure as study one (see also Appendix A, and I,). The main difference was in the quantity of media needed from study to study. Table 6 shows the translation of media bottles to the specific quantity (in ml) of media used in each study.

Table 7. List of Sterilized Materials

<u>EtO Autoclaved Materials</u>	
<u>Study Two</u>	<u>Study Three</u>
1 ePTFE Graft	4 ePTFE Grafts
3 Forceps	3 Forceps
4 Scissors	4 Scissors
Degassing Media Flask	Degassing Media Flask
3-4 500 ml Media Bottles	5-6 500 ml Media Bottles
<u>Study Two</u>	<u>Study Three</u>
2 Bioreactor Chambers	4 Bioreactor Chambers
Chamber Fittings	Chamber Fittings
2 Media Reservoir Tubing Sets	4 Media Reservoir Tubing Sets

Table 8. Media Quantities Required for Study Two and Study Three (1 bottle = 500 ml)

Media	Study Two	Study Three
Conditioning Media	1 bottle	2 bottles
Bioreactor Media	3 bottles	3 bottles
HUVEC Media	1 bottle	2 bottles

PART II: BVM Protocol: Vessel 1 Preparation

1. Wash hands, spray down gloved hands with IPA, spray down the hood, and maintain aseptic area.
2. Retrieve Bioreactor media and conditioning media, spray down with IPA, and bring into hood.
3. Bring in sterilized packaged bioreactor chamber under hood. Pull out chamber from package by tugging on outside valve (Do not ever run hands over sterile chamber body once opened).
4. Fill chamber with bioreactor media, go just over the fitting level.
5. Bring in sterilized packaged ePTFE grafts into hood. Open package carefully and dump graft into bioreactor chamber (1 graft per chamber). Do not let graft fall onto hood surface or nonsterile area.

6. Obtain sterile-sterile gloves from cabinet. Insert right hand first carefully, use left hand to navigate the non-sterile bottom portion of the glove over right hand. Then carefully use your gloved right hand to navigate left hand properly into glove. Avoid any contact w/ nonsterile surfaces during this process!
7. Insert fingers into chamber and secure grafts into fittings. Twist the barbed fittings onto the female ends of the grafts, not the other way around.
8. Dispose of sterile-sterile gloves and put on regular latex gloves. Obtain sterilized packaged bioreactor chamber lids and open under sterile hood; again avoid contacting inner surface of the lid, instead use the lid flaps to grip the lid and navigate it onto the bioreactor chamber, snap the lid closed.
9. Prepare reservoir chambers; obtain packaged reservoir tubing/pump tubing along with clean 50 ml media vials (1 per package). Open tubing package under hood. Assemble accordingly to optimize distance of pump tubing relative to peristaltic pump (see diagram). *Brown pump tubing should be attached to the inlet port side of the media vial cap.
10. Fill media vial w/ 40 ml of conditioning media (CM).
11. Allow CM to prime the tubing by opening the valves on the tubing and turning the media vial upside down.
12. When it is seen that CM has flowed all the way back to the media vial, clamp off both ends of tubing. Set aside media vials w/ properly attached tubing until the conditioning stage.
13. Now it's time to prime the attached ePTFE graft with CM.
14. Obtain two troughs per ePTFE graft being primed (per bioreactor chamber), one for catching the fluid from the exit port, the other trough is used to hold the CM at the entrance port as you suction it up with a syringe pump.

15. Fill “inlet” trough with 6-7 ml of CM, and use syringe pump to suck up all the media.
16. Before injecting CM with syringe pump, it is crucial to have valve orientations opened in the following order: inlet stopcock (white) pointed away /opposite (left) from fluid flow, exit valve open (turned parallel to tubing), and lumen valve open/transmural valve closed.
17. Insert syringe into inlet port and start slowly applying constant pressure on syringe; when media in syringe reaches 1-2 ml, close lumen valve to allow flow transmurally, and apply pressure to push remaining fluid transmurally through graft. STOP pushing on the syringe right before the last bit of media is pushed through to avoid pushing air through the tubing and into the ePTFE graft pores.
18. Now close off transmural valve, exit valve, and inlet valve to avoid spillage (in preparation for the next step).
19. Now the assembled bioreactor with the primed ePTFE graft is ready for its first conditioning pump run.
20. Bring in peristaltic pump beforehand into the large incubator; spray down with IPA before doing so.
21. Transfer assembled bioreactor from underneath the hood and into the large incubator as well as the media reservoir assembly.
22. Assemble bioreactor setup with media reservoir and tubing appropriately onto the peristaltic pump. **Make sure gray tubing part is fitted into the pump trays correctly. This tubing should be attached to so that small intermediate tubing attaches to the inlet port of the bioreactor chamber.
23. Now, with setup complete, open lumen and exit valve and make sure inlet stopcock is oriented upwards so as to flow through the tubing. (Transmural valve should be closed initially).

24. Turn on peristaltic pump and then push start button. Run pump at high speeds (150 RPMs) for 10 minutes for luminal conditioning. Make sure you see a drip in the media chamber. After 10 minutes of pumping, stop pump.
25. Now allow transmural flow conditioning (by closing off lumen valve). Start pump again w/ transmural flow and run pump at slightly lower speeds for 5 minutes (70-100 RPMs). 80 rpms was the operating speed.
26. Stop pump, close off all valves, clamp off media reservoir tubing to avoid dripping media everywhere during the transfer stage of the bioreactor setup back underneath the hood.
27. Now ready for cell sodding stage.

PART III: Cell Sodding and Incubation

28. Obtain your culture flasks (T225s) from small incubator, check cell confluency prior to sodding.

Observations:

- **Vessel 1: 60%**

29. Use standard cell culture techniques to isolate cells to eventually get a pellet of HUVECs.
30. First, aspirate out media from T225 flask, rinse w/ DCF-PBS, aspirate out again, than add 6 ml of trypsin-EDTA (this amount can vary) to flask. Then added the same amount of CM (as trypsin used) to detrypsinize. Pipette solution up and down a few times to make sure most of the cells are captured. Beat flask with palm of hand for 5-10 minutes to further release cells (Check cells again under microscope to reaffirm presence of cells in media and that the trypsin worked).
31. Pipette media from flask into a 50 ml media vial/conical.

32. Place conical inside centrifuge machine.
33. Balance conical w/ another conical (could be cell media conical or just plain water).
34. Run centrifuge for approximately 4 minutes on setting 4.
35. Once complete, take conical out slowly to avoid dispersing pellet back into solution, and bring conical into hood!
36. Now aspirate fluid from vial carefully, making sure not to suck up the pellet!
37. Now resuspend pellet with 3 ml of HUVEC/EC media per bioreactor chamber. If you plan on sodding multiple grafts with a single pellet, (for example if you want to sod 2 grafts, resuspend pellet with 6 ml of HUVEC media, and evenly divide the HUVEC cell solution per chamber/3 ml per graft).
38. With 10 ml pipette suck up all of the media with dispersed pellet, make sure the entire pellet has been resuspended.
39. Now obtain two troughs again per bioreactor, add the crucial sucked up cell solution into the inlet trough located in front of the inlet port.
40. Try and maintain sterility here; avoid running hands over trough once cell media has been dispersed.
41. Now with media in trough, ready to sod. Once again make the valves are oriented are correctly.
42. Lumen valve should be closed!! Exit valve should be open and be set on top of the exit trough. Inlet port should be pointed away from graft (left) so that cells flow into ePTFE graft.
43. Using a clean syringe, suck all media, avoid sucking up air.

44. Attach syringe to inlet port, slowly apply pressure: stop applying pressure near the end to avoid pushing air bubbles through the ePTFE graft pores!
45. Once the graft has been sodded, “chase” the HUVEC cell solution injected by injecting 3 ml of HUVEC media into graft to ensure that the HUVECs were properly placed inside the interstices of the ePTE grafts.
46. Now ready to transfer bioreactor setup back into large incubator and connect to pump and media reservoir (media reservoir should be switched out so it contains fresh HUVEC media instead of the previous CM media!).
47. Close off exit valve and inlet stopcock to avoid spillage of media during transfer.
48. Reassemble bioreactor system: bioreactor chamber to media reservoir and media reservoir tubing into pump and back into the inlet port.
49. Now start pump at very low speeds: Maintain 10 rpm transmural valve flow for 1/ 2 hr.
50. Once a consistent circular flow has been established close incubator door and turn on CO₂ tank (Not Necessary for 24hr cultures).
51. After ½ hr, come back and open lumen valve (thus disallowing transmural flow).
52. Ramp up flow speeds accordingly: constant 15 rpm for additional hour.
53. Peristaltic pump was shut down at this junction; vessel was cultured in static condition.
54. Take down/fix vessel 24 hours later.

Scaffold Fixing of BVM One for Study Two

Since there were no flow ramp-ups for the studies contained within this chapter, the critical closing point came twenty-four hours later when the BVM was taken down

from the bioreactor culture system, and stored in 5 ml of glutalderhyde. The pump was turned off to allow the bioreactor chamber and media reservoir to be removed from the peristaltic pump. Finally, the vessels were put inside a small vial containing prepared glutalderhyde. The vial was labeled with the time of take down and what ends of the graft were the distal and proximal end with respect to the position it was in within the bioreactor chamber.

Appendix C – Study Three BVM Setup - Summer '10: Sept. 1st, 2010

PART I: Culture Process

Thawing HUVECs

After thawing the HUVECs in the warm water bath, the cells were transferred into a T75 precoated with 12 ml of HUVEC media at 37°C. The session concluded by returning the T75 containing HUVECs back into the Shel Lab incubator at normal cultural conditions.

Passing Stages

The third study consisted of three separate passages that would develop the P4 HUVECs eventually into a P7 expansion. The first passing stage concluded with transferring the HUVECs from the single T75 into two newly prepared T75s with 12 ml of HUVEC media in each. Cell confluency before passing was recorded at 90 % upon observation using the CKX31 Optical Microscope. Different from the previous studies, 8 ml of trypsin was used to dislodge the HUVECs followed by incorporation of 8 ml of HUVEC media for deactivation. Later the HUVEC media solution was transferred evenly into each of the two T75s before being returned into the incubator.

After 48 hours each T75 flask in study 3 was reevaluated for confluency. In most circumstances if the cells did not appear very confluent (over 70 %), an additional 24 hours of incubation was maintained to improve cell proliferation and expansion. However, at the time of recording each T75 flask appeared near 85% confluency. Since the HUVECs appeared confluent, the goal of expanding the two T75s into four T75s was carried forward.

With the T75s in incubation, the final passing stage consisted of passing each individual T75 into its own T225. The end result was a 1 to 3 pass that would convert the four P6 T75s into four P7 T225s. Confluency was recorded between 50-60 % amongst the four flasks; a figure that was lower than expected. Hence, the passage was carried in hopes that the cells would proliferate adequately over the 72 hours up until the time of cell sodding on BVM setup day.

The passage was carried out following standard cell passaging protocol. Two of the four T75s were removed at a time from the incubator and brought into the flow hood; upon which the HUVECs would be passed into the prepared prewarmed T225s. Once the four T225s successfully contained the P7 HUVECS, the remaining four T75s were disposed of and all remaining media was returned to proper refrigeration.

Feed Cells

The 4 T75s in study 3 were refreshed with HUVEC media in the same manner as in the study 2. Furthermore the quantity of HUVEC media replenished changed from 36 ml to 12 ml per flask based on the fact that T75s were being used in this situation and not the larger T225s. The only other feeding stage for study 3 came after the third and finally passing stage. In that instance 4 T225s were replaced with 36 ml of HUVEC media per flask. At that point the cells were ready for the final passing stage and examination of HUVEC confluency after 24 hours.

Preparation of ePTFE Grafts

ePTFE grafts, 3.5 cm long and 2 mm in diameter were used as the BVM scaffold structures for study two and study three from Bard incorporation. Similar to study one,

the graft material was mounted onto barbed fittings and then sutured around the fitting for proper closure and insulation. Once the graft was prepped, it was inserted into an autoclave package with an indicator strip and autoclaved for 30 min at 250° F with an additional 30 minutes for drying inside the Ritter M9 Autoclave. The graft was later retrieved before implantation inside the BVM model where they were denucleated and conditioned in a series of treatments in graded ethanols and CM.

The main difference of the denucleation procedure between study two and study three came from the increased amount of degrading ethanol vials needed. Since four vessels were prepared in this study, an additional set of 10 ml vials of appropriate alcohols and CM was made. No deviations were made in regards to the time the ePTFE grafts were exposed to each alcohol; also 40 ml of CM was degassed in the depressurizing flask for the four grafts (10 ml per graft). The result ended in four denucleated vessels placed inside the Shel Lab incubator at 37°C ready for setup day.

Materials and Sterilization

The bioreactor (BR), HUVEC, and Conditioning Media (CM) used in study three contain the same components as mentioned in the previous two studies. So forth, the preparation in making the medias also followed the same procedure as mentioned in Appendix A, and I.

PART II: BVM Protocol: Vessel 1 and Vessel 2 Preparation

1. Wash hands, spray down gloved hands with IPA, spray down the hood, and maintain aseptic area.
2. Retrieve Bioreactor media and conditioning media, spray down with IPA, and bring into hood.
3. Bring in sterilized packaged bioreactor chamber under hood. Pull out chamber from package by tugging on outside valve (Do not ever run hands over sterile chamber body once opened).
4. Fill chamber with bioreactor media, go just over the fitting level.
5. Bring in sterilized packaged ePTFE grafts into hood. Open package carefully and dump graft into bioreactor chamber (1 graft per chamber). Do not let graft fall onto hood surface or nonsterile area.
6. Obtain sterile-sterile gloves from cabinet. Insert right hand first carefully, use left hand to navigate the non-sterile bottom portion of the glove over right hand. Then carefully use your gloved right hand to navigate left hand properly into glove. Avoid any contact w/ nonsterile surfaces during this process!
7. Insert fingers into chamber and secure grafts into fittings. Twist the barbed fittings onto the female ends of the grafts, not the other way around.
8. Dispose of sterile-sterile gloves and put on regular latex gloves. Obtain sterilized packaged bioreactor chamber lids and open under sterile hood; again avoid contacting inner surface of the lid, instead use the lid flaps to grip the lid and navigate it onto the bioreactor chamber, snap the lid closed.
9. Prepare reservoir chambers; obtain packaged reservoir tubing/pump tubing along with clean 50 ml media vials (1 per package). Open tubing package under hood. Assemble accordingly to optimize distance of pump tubing relative to peristaltic

pump (see diagram). *Brown pump tubing should be attached to the inlet port side of the media vial cap.

10. Fill media vial w/ 40 ml of conditioning media (CM).
11. Allow CM to prime the tubing by opening the valves on the tubing and turning the media vial upside down.
12. When it is seen that CM has flowed all the way back to the media vial, clamp off both ends of tubing. Set aside media vials w/ properly attached tubing until the conditioning step.
13. Now it's time to prime the attached ePTFE graft with CM.
14. Obtain two troughs per ePTFE graft being primed (per bioreactor chamber), one for catching the fluid from the exit port, the other trough is used to hold the CM at the entrance port as you suction it up with a syringe pump.
15. Fill "inlet" trough with 6-7 ml of CM, and use syringe pump to suck up all the media.
16. Before injecting CM with syringe pump, it is crucial to have valve orientations opened in the following order: inlet stopcock (white) pointed away /opposite (left) from fluid flow, exit valve open (turned parallel to tubing), and lumen valve open/transmural valve closed.
17. Insert syringe into inlet port and start slowly applying constant pressure on syringe; when media in syringe reaches 1-2 ml, close lumen valve to allow flow transmurally, and apply pressure to push remaining fluid transmurally through graft. STOP pushing on the syringe right before the last bit of media is pushed through to avoid pushing air through the tubing and into the ePTFE graft pores.
18. Now close off transmural valve, exit valve, and inlet valve to avoid spillage (in preparation for the next step).

19. Now the assembled bioreactor with the primed ePTFE graft is ready for its first conditioning pump run.
20. Bring in peristaltic pump beforehand into the large incubator; spray down with IPA before doing so.
21. Transfer assembled bioreactor from underneath the hood and into the large incubator as well as the media reservoir assembly.
22. Assemble bioreactor setup with media reservoir and tubing appropriately onto the peristaltic pump. **Make sure gray tubing part is fitted into the pump trays correctly. This tubing should be attached to so that small intermediate tubing attaches to the inlet port of the bioreactor chamber.
23. Now, with setup complete, open lumen and exit valve and make sure inlet stopcock is oriented upwards so as to flow through the tubing. (Transmural valve should be closed initially)
24. Turn on peristaltic pump and then push start button. Run pump at high speeds (200 RPMs) for 10 minutes for luminal conditioning. Make sure you see a drip in the media chamber. After 10 minutes of pumping, stop pump.
25. Now allow transmural flow conditioning (by closing off lumen valve). Start pump again w/ transmural flow and run pump at slightly lower speeds for 5 minutes (70-100 RPMs). 70 rpms was operating speed.
26. Stop pump, close off all valves, clamp off media reservoir tubing to avoid dripping media everywhere during the transfer stage of the bioreactor setup back underneath the hood.
27. Now ready for cell sodding stage.

PART III: Cell Sodding and Incubation

28. Obtain your culture flasks (T225s) from small incubator, check cell confluency prior to sodding.

Observations:

- **Vessel 1: 60%**
- **Vessel 2: 60 - 70%**
- **Vessel 3: 80 - 90%**
- **Vessel 4: 80 - 90%**

29. Use standard cell culture techniques to isolate cells to eventually get a pellet of HUVECs.

30. First, aspirate out media from T225 flask, rinse w/ DCF-PBS, aspirate out again, than add 8 ml of trypsin-EDTA (this amount can vary) to flask. Then add the same amount of CM (or greater amount than trypsin used) to detrypsinize. (Use BR media if CM is low). Pipette solution up and down a few times to make sure most of the cells are captured. Beat flask with palm of hand for 5-10 minutes to further release cells (Check cells again under microscope to reaffirm presence of cells in media and that the trypsin worked).

31. Pipette media from flask into a 50 ml media vial/conical.

32. Place conical inside centrifuge machine.

33. Balance conical w/ another conical (could be cell media conical or just plain water).

34. Run centrifuge for approximately 4 minutes on setting 4.

35. Once complete, take conical out slowly to avoid dispersing pellet back into solution, and bring conical into hood!

36. Now aspirate fluid from vial carefully, making sure not to suck up the pellet!

37. Now resuspend pellet with 3 ml of HUVEC/EC media per bioreactor chamber. If you plan on sodding multiple grafts with a single pellet (for example if you want to sod 2 grafts, resuspend pellet with 6 ml of HUVEC media, and evenly divide the HUVEC cell solution per chamber/3 ml per graft).
38. With 10 ml pipette suck up all of the media with dispersed pellet, make sure the entire pellet has been resuspended.
39. Now obtain two troughs again per bioreactor, add the crucial sucked up cell solution into the inlet trough located in front of the inlet port.
40. Try and maintain sterility here; avoid running hands over trough once cell media has been dispersed.
41. Now with media in trough, ready to sod. Once again make the valves are oriented are correctly.
42. Lumen valve should be closed!! Exit valve should be open and be set on top of the exit trough. Inlet port should be pointed away from graft (left) so that cells flow into ePTFE graft.
43. Using a clean syringe, suck all media, avoid sucking up air.
44. Attach syringe to inlet port, slowly apply pressure: stop applying pressure near the end to avoid pushing air bubbles through the ePTFE graft pores!
45. Once the graft has been sodded, "chase" the HUVEC cell solution injected by injecting 4 ml of HUVEC media into graft to ensure that the HUVECs were properly placed inside the interstices of the ePTE grafts.
46. Now ready to transfer bioreactor setup back into large incubator and connect to pump and media reservoir (media reservoir should be switched out so it contains fresh HUVEC media instead of the previous CM media!).

47. Close off exit valve and inlet stopcock to avoid spillage of media during transfer.
48. Reassemble bioreactor system: bioreactor chamber to media reservoir and media reservoir tubing into pump and back into the inlet port.
49. Now start pump at very low speeds: Maintain 10 rpm transmural valve flow for one hour.
50. Once a consistent circular flow has been established close incubator door and turn on CO₂ tank.
51. After one hour, come back and open lumen valve (thus disallowing transmural flow).
52. Ramp up flow speeds accordingly: 10 rpm for additional 24 hours.
53. Repeat process for other vessel(s) being sodded.
54. Come back on scheduled predetermined days to ram up flow (If Necessary) accordingly and eventually take down/fix vessels. (Leaks Present in first two vessels)

PART IV: BVM Protocol 2: Vessels 3 and 4

- 1-47. Same as BVM protocol 1.
48. Maintain 15 rpm transmural valve flow for 1 hr.
49. After 1 hour, pause the pump, and switch to luminal flow while keeping the same flow speed.
50. Ramp up flow speeds accordingly: Maintain at 15 rpm for additional 24 hours.

Scaffold Fixing of BVMs One through Four for Study Three

Twenty-four hours later the 4 BVMs were taken down from the bioreactor culture conditions and stored in 10 ml of glutalderhyde until BBI and histology imaging. BVMs 1 and 2 were first removed from the bioreactor chambers once the peristaltic pump was turned off. The BR media contained in chamber 1 showed signs of continuous of leakage from the connection points of the bioreactor valves. Furthermore, BVMs 3 and 4 were removed without any notable discrepancies in the setup except that the BR media had an alteration in color, an indication of possible pH flux. Each BVM was removed and preserved in glutalderhyde until further processing. All vials were appropriately labeled with the time of take down, and the distal and proximal ends of the vessels.

Appendix D – Histology Protocol: Study One and Study Two

1. Obtain sectioned slides desired for staining from appropriate slide casing.
2. Warm up wax cover on slide until wax is clear; approximately 8-10 minutes.
3. Cool down slides until some of the wax is observable again from its present translucent state.
4. Staining Procedure is as follows.
 - a. 3-min: Xylene
 - b. 1-min: Air Dry
 - c. 2-min: 100% EtOH
 - d. 2-min: 95% EtOH
 - e. 20-min: Air Dry
 - f. 4-min: Hematoxylin
 - g. 1-min: Water Distilled
 - h. 30 sec: Clarifier
 - i. 1 min: Bluing
 - j. 1 min: 95% EtOH
 - k. 80 sec: Eosin
 - l. 1 min: 100% EtOH
 - m. 1 min: Air Dry
 - n. 1 min: Xylene

5. Stay gentle when transferring slides from one reagent to the next.
6. After staining is complete, apply glue droplets to specific area of the slide, with other hand use forceps to cover slide with coverslip; avoid using too much glue for adhesion to avoid interference with stained vessel cross-section.

Appendix E - Histology Protocol Study Three

1. Obtain sectioned slides desired for staining from appropriate slide casing.
2. Warm up wax cover on slide until wax is clear; approximately 8-10 minutes.
3. Cool down slides until some of the wax is observable again from its present translucent state.
4. Staining Procedures are as follows.
 - a. 3-min: Xylene 1
 - b. 3-min: Xylene 2
 - c. 1-min: Air Dry
 - d. 2-min: 100% EtOH
 - e. 2-min: 100% EtOH
 - f. 2-min: 95% EtOH
 - g. 2-min: Air Dry
 - h. 4-min: Hematoxylin
 - i. 1 min: Distilled H₂O
 - j. 30 sec: Clarifier
 - k. 1 min: Bluing
 - l. 1 min: 95% EtOH
 - m. 90 sec: Eosin
 - n. 1 min: 100% EtOH

- o. 1 min: Air Dry
 - p. 1 min: Xylene 1
 - q. 1 min: Xylene 2
5. Stay gentle when transferring slides from one reagent to the next.
 6. After staining is complete, apply glue droplets to specific area of the slide, with other hand use forceps to cover slide with coverslip; avoid using too much glue for adhesion to avoid interference with stained vessel cross-section.

Appendix F – HUVEC Cell Average Counts (Study Two and Three)

Study Two

Vessel 1 – Proximal and Mid-Distal Counts

Figure 45: Here the BVM vessel at the proximal region shows consistent yet lower cell coverage relative to the mid-proximal regions from the vessels in study 1. The cell coverage was observed in this region was $4\text{-}5 \times 10^6$ cells/cm².

Figure 50: A mid-distal BBI shot of the BVM. Due to the bending of the scaffold, the outer edges appear unfocused. The cell average count is lower and more inconsistent in this region with respect to the mid-proximal area presented in chapter 2. On average the areas of the grid indicate cell count is around $3\text{-}4 \times 10^6$ cells/cm².

Study Three

Vessel 1 – Mid-Distal Counts

Figure 64: Shows vessel 1 at the distal end of inner wall with minimal cell coverage with a cell density of $0 - 1 \times 10^6$ cells/cm². The unfocused area of the image did not allow for an accurate assessment of the cell coverage in that location of the vessel.

Vessel 2 – Proximal Count

Figure 68: A few visible cells scattered in the proximal area of the vessel fragment with an average cell density of 0.2×10^6 cells/cm².

Vessel 3 – Mid-Proximal and Distal Counts

Figure 71: Shows the lack of continuity of HUVEC presence across the ePTFE graft near the proximal region. Again counts here ranged from $1-2 \times 10^6$ cells/cm².

Figure 70: HUVEC presence across the ePTFE graft near the proximal region. Cell adhesion presence for vessel 3 was a bit more prevalent than the first two vessels but still averaged around $1-2 \times 10^6$ cells/cm².

Vessel 4 – Proximal and Mid-Distal Counts

Figure 75: Shows the continuity of HUVEC presence across the ePTFE graft near the proximal region with a much higher cell average count than the previous vessels of study 3. An average of the three areas gives a cell average around $5\text{-}6 \times 10^6$ cells/cm².

Figure 79: Here the bottom area of the mid-distal region of the vessel is flattened out with a pair of forceps to assist in impeding the graft from curving upwards too much. A heavy cell population can be seen along the cut area of the graft with areas that were to numerous count individual cells, but an approximation gives an average around 7×10^6 cells/cm².

Appendix G – BBI (Bisbenzimidazole Imaging) Protocol

1. Wrap nonsterile 15 mL conicals in foil. Thoroughly label conicals for all samples. Also, keep track of sample orientations with respect to proximal and distal ends inside the conical.
2. Make BBI solution: Always keep stock solution wrapped in foil.
 - a. Use small tube of stock and make a 1:1000 (10 μ m in 10 dilution with milli-Q-water.
 - b. Mix by inverting.
3. Cut sample with blade, be careful to not disrupt cell lining inside lumen. Avoid squeezing, touching, and scraping as much as possible.
4. Use washed forceps to place samples in corresponding 15 ml conicals; allow samples to soak for at least 15 minutes inside the BBI solution.
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.
 - a. Log into Notebook.
 - b. Set microscope to turret number four; the wide horizontal dial below eyepiece.
 - c. Turn on Olympus lamp located to the right of the computer monitor.
 - d. Turn on optiscan wheels; a green switch to right of Olympus lamp.
 - e. Turn on camera switch located on the top of the microscope.

- f. Set filter wheel 1 to 1, also set filter wheel 2 to 1 on prior keypad at located at the microscope base.
 - g. Open shutter.
 - h. Set prior keypad to shutter one.
 - i. Sign into computer by clicking Dr. Cardinal's account and enter correct password.
 - j. Click Q capture pro located on the desktop.
 - k. Dial objectives to desired magnification.
 - l. Set thin bar below eyepiece on the microscope to the icon of an eye and camera.
 - m. Increase number for lighter images; decrease number for darker images.
 - n. If scaffolds or vessel conduits are too wet, blot the end of the scaffold with a Kimwipe.
 - o. Take desired amount pictures across the length of the vessel; select capture or autocapture.
 - p. Save Pictures.
- 8. Shut down system by switching Olympus lamp off, as wells as the optiscan wheels and camera switch.
 - 9. Log out of notebook.

Appendix H - Microbiological Testing Protocols

BVM Microbiological Assessment Protocols

Creating a New SOP

Purpose: To provide general guidelines for creating a new Standard Operating Procedure (SOP).

Safety Precautions: N/A

Supplies: N/A

Remarks: SOPs should be given a distinct title before approval by Dr. Cardinal to prevent creation of duplicate SOP topics to ensure the SOPs are current with lab practices.

Procedure:

1. After determining the necessity for a new SOP, create a draft copy of the SOP to be issued following the format: Purpose, Safety Precautions, Supplies, Media/Reagents, Procedure, and References. One or more of the sections may not be applicable to a new SOP.
2. Submit an electronic copy to Dr. Cardinal. Dr. Cardinal will check the formatting and issue a SOP final copy with appropriate number or designation if no changes are required.
3. If changes are necessary, Dr. Cardinal will return the SOP to the preparer to incorporate any adjustments; the preparer will then resubmit the SOP to Dr. Cardinal for final review.
4. If no changes are needed the preparer and reviewer will sign and print name on the original SOP document. Dr. Cardinal will provide copies and keep master copy.

Note: Working copies of SOPs should be revised and submitted only electronically to Dr. Cardinal for further review.

References: "Creating a New SOP" by Tony Piazza, 2008 QA/QC

Aerobic Plate Count (APC) Petrifilm

Purpose: To ensure proper use of Aerobic Plate Count Petrifilm.

Safety Precautions:

1. Always wear gloves and appropriate lab attire when plating samples.
2. Wash hands before and after plating samples and handling Petrifilm.
3. Dispose of all plated materials and plates in the biohazard waste upon completion of analysis.

Supplies:

Incubator $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$
Refrigerator $\leq 8^{\circ}\text{C}$
3M APC Petrifilm
Sterile Gloves

Sterile Pipets or Pipet tips
Petrifilm Spreader
Quebec Colony Counter (optional)

Remarks:

1. Do not use Petrifilm after the expiration date; use the oldest lots of Petrifilm first.
2. APC is also known as Total Plate Count (TPC), Standard Plate Count (SPC) or Heterotrophic Plate Count (HPC).
3. All plating work should be performed within the laminar flow hood.
4. Unopened packages of Petrifilm must be stored in the refrigerator at $\leq 8^{\circ}\text{C}$.
5. If a package is opened, seal the remaining unused portion by the folding the end over and taping shut (do refrigerate package once opened).
6. DO NOT USE buffers containing citrate, bisulfate, or thiosulfate; these chelating agents and antioxidants can inhibit microbial growth.
7. Adjust pH of samples between 6.6 and 7.2 before plating on Petrifilm. For acidic products use 1N NaOH, for alkaline products use 1N HCL.

Procedure:

Inoculation

1. Prepare sample and materials underneath laminar flow hood accordingly.
2. Place petrifilm plate on a level surface and lift top of film.
3. Holding pipet perpendicular to the plate, place 1 mL of drawn sample onto the center of the bottom film.
4. Release top film and allow it to drop. Do not roll top film down.
5. With ridge side down, place the spreader on top of film over inoculum (sample).
6. Gently apply pressure on spreader to distribute inoculums over circular area defined by spreader. Do not twist or slide spreader.
7. Lift spreader and wait at least one minute for gel to form. The Petrifilm is now ready for incubation.

Incubation

1. Label Petrifilm accordingly and place Petrifilm in incubator, clear side up, in stacks of 10 or less.
2. Incubate at $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 48 hours.

Interpretation and Identification of Aerobic Microorganisms

1. Colonies on Petrifilm will mostly appear as red colonies, regardless of size or color intensity.
2. Count each speck as a Colony Forming Unit (CFU).
3. The preferable counting range on an APC Petrifilm is 25-250 colonies.
4. If the colony count is more than 250, estimate the count. Determine the average number of colonies in one square centimeter (1 cm^2) and multiply it by 20 to obtain the total count per plate.
5. Report results as CFU/g for products (media) and CFU/50 sq cm for equipment/swab contact surfaces and CFU/ sq m for sponges.

References: 3M Aerobic Plate Count Petrifilm Package Insert

Coliform Count & E.Coli Plate Count - Petrifilm

Purpose: To ensure the proper use of Total Coliform and E. coli Petrifilm

Safety Precautions:

1. Always wear gloves and appropriate lab attire when plating samples.
2. Wash hands before and after plating samples and handling Petrifilm.
3. Dispose of all plated materials and plates in the biohazard waste upon completion of analysis.

Supplies:

Incubator $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$
Refrigerator $\leq 8^{\circ}\text{C}$
3M APC Petrifilm
Sterile Gloves

Sterile Pipets or Pipet tips
Petrifilm Spreader
Quebec Colony Counter (optional)
pH meter (optional)

Remarks:

1. Do not use Petrifilm after the expiration date, use the oldest lots of Petrifilm first.
2. All plating work should be performed within the laminar flow hood.
3. Unopened packages of Petrifilm must be store in the refrigerator at $\leq 8^{\circ}\text{C}$.
4. If a package is opened, seal the remaining unused portion by the folding the end over and taping shut (do refrigerate package once opened).
5. DO NOT USE buffers containing citrate, bisulfate, or thiosulfate; they can inhibit growth.
6. Adjust pH of samples between 6.6 and 7.2 before plating on Petrifilm. For acidic products use 1N NaOH, for alkaline products use 1N HCL.
7. DO NOT USE this plate alone for the detection of *E. coli* 0157:H7. Like most other E.coli/Coliform media, this plate will not specifically indicate the presence of the 0157 strain.

Procedure:

Inoculation

1. Prepare sample and materials underneath laminar flow hood accordingly.
2. Place Petrifilm plate on a level surface and lift top of film.
3. Holding pipet perpendicular to the plate, place 1 mL of drawn sample onto the center of the bottom film.
4. Release top film and allow it to drop. Do not roll top film down.
5. With ridge side down, place the spreader on top of film over inoculum (sample)
6. Gently apply pressure on spreader to distribute inoculum over circular area defined by spreader. Do not twist or slide spreader.
7. Lift spreader and wait at least one minute for gel to form. The Petrifilm is now ready for incubation.

Incubation

1. Place Petrifilm in incubator, clear side up, in stacks of 10 or less.
2. Incubate at $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 48 hours.
3. Total Coliforms can be read at 24 hours.

Interpretation and Identification of Total Coliform (TC)

1. Coliform colonies will appear as red and/or blue colonies with gas production (corresponding gas bubbles) surrounding the colonies. If gas production is not present it is not a coliform.
2. Each colony with gas is considered a Colony Forming Unit (CFU).
3. The preferable counting range on an EC/Coliform Plate is 25-250 colonies.
4. If the colony count is higher than 250, count at least three individual grids and average the number and multiply by 20 to obtain the total count per plate.
5. Report results as CFU/g for products (culture media), CFU/50 sq cm for swab samples and CFU/sq m for sponge samples.

Interpretation and Identification of E. coli (EC)

1. E. coli colonies will appear as blue colonies with gas production (corresponding gas bubbles) surrounding the colonies. If gas production is not present it is not E. coli.
2. Each blue colony with gas is considered a Colony Forming Unit (CFU).
3. The preferable counting range on an EC/Coliform Plate is 25-250 colonies.
4. If the colony count is higher than 250, count at least three individual grids and average the number and multiply by 20 to obtain the total count per plate.
5. Report results as CFU/ml for cultural media or water, CFU/50 sq cm for swab samples and CFU/sq m for sponge samples.
6. E. coli colonies can be confirmed through further AOAC SOPs as directed.

References: 3M E.coli/Coliform Count Petrifilm Package Insert and Interpretation Guide

Sterility Testing of Glassware, Equipment, and Media

Purpose: To assure the sterility of laboratory glassware, equipment, and media.

Safety Precautions:

1. Wash hands before and after performing microbiology procedures.
2. Dispose of media used for sterility checks in biohazard waste.

Supplies:

Aerobic Plate Count Petrifilm
Pipets
Sharpie Marker
Incubator

Reagents:

Sterile Phosphate Buffer Solution
Non-selective Media

Sterile Dilution Water
Fluid Thioglycolate Broth

Remarks:

1. Always log in results of sterility testing in the QA/QC notebook.
2. Perform sterility testing on each new batch of culture media made.

Procedure:

Rinse Filtration Technique for Utensils, Containers, and Media Bottles

1. Fill the container to be tested approximately one quarter of the way full with sterile phosphate buffer solution.
2. Cap the container and shake it thoroughly so the buffer solution coats all parts of the container.
3. Continue to shake for 2-3 minutes.
4. Pipet out 1 ml of buffer onto an aerobic plate count Petrifilm.
5. Place Petrifilm in incubator at 35°C for 24 hrs.
6. While incubating test film, place a blank aerobic plate count Petrifilm with 1 ml of sterile phosphate buffer solution under the same test conditions to confirm no growth on blank film.
7. After incubation, remove Petrifilm and check results. Record results in appropriate QA/QC notebook.

Sterility Testing of Media and Media Bottles

1. Randomly choose 4 sterile media bottles from the laboratory supply and divide them into two sets of 2 bottles in each set.
2. Pour 25 ml of one culture media into the bottle, label bottle.
3. Place bottle in incubator at 35°C for 24 hrs.
4. After the completion of incubation, plate 25 ml of warmed media into 225 ml of BFB media. Shake thoroughly.
5. Repeat process with DI water in TE lab or PBS that is used in cell passages.
6. Plate 1 ml of diluted media/BFB solution onto an APC Petrifilm.
7. Incubate Petrifilm at same conditions for an additional 24 hrs.
8. No growth should be seen on Petrifilm.

Sterility Testing of Culture Flasks Culture/Test Tubes

1. Randomly select 3 sterile tubes from the laboratory supply.
2. Fill tubes ½ full with fluid thioglycolate.
3. Incubate tubes at 35°C for 24 hrs.
4. No growth should be present at the end of the incubation period.

References: "Compendium of Methods for the Microbiological Examination of Foods,"
3rd ed., 1992

Swab Contact Method

Purpose: To ensure proper sampling procedures for presence of Aerobic microorganisms, total coliforms, and E.coli through the swab contact method.

Safety Precautions:

1. Wash hands before and after performing microbiology procedures.
2. Dispose of swab tube after plating in biohazard waste.

Supplies:

Letheen Swab Tubes
Water Resistant Marker
Sterile Gloves

Remarks:

1. Do not open the swab tube until you start sampling.
2. Samples should be taken just prior to lab analysis or should begin within 24 hours of sampling.

Procedure:

1. Select equipment, table or hood surface, floor, wall or any used object in the TE lab.
2. For reference, mark outside of the sterile vial with surface swabbing description.
3. Put on a new pair of rubber gloves.
4. Open swab vial and carefully remove swab without touching the swab end.
5. Hold the swab handle to make a 30° angle contact with the surface.

6. Rub the swab end slowly and thoroughly over approximately 50 cm² of surface three times, reversing the direction between strokes.
7. Return the swab to the solution vial, rinse briefly in the solution.
8. Swab four more 50 cm² areas of the SAME surface/object being sampled, as above, rinsing the swab in the solution after each swabbing.
9. After all areas of the surface or object have been swabbed, return the swab to the vial, ensuring the cap is tight.
10. Repeat steps 1 through 9 above for each additional surface to be swabbed.
11. Samples need to be analyzed within 24 hours.
12. If sample is not immediately processed, place sample(s) into a cooler or fridge to maintain sample condition over time.

Plating

1. After sample is obtained, remove swab from tube and carefully hold with one hand while preventing swab tip from touching tube or hand.
2. Using 10 ml pipet, carefully transfer 1 ml of media sample onto appropriate EC/TC and or APC Petrifilm. Label Petrifilm with sample area description.
3. Transfer Petrifilm into 35°C incubator for 48 hours for further analyses.

References: "Compendium of Methods for the Microbiological Examination of Foods,"
3rd ed., 1992

Environmental Sponge Sampling Procedure

Purpose: To ensure proper sampling procedures for presence of Aerobic microorganisms, total coliforms and E.coli through the sponge contact method.

Safety Precautions:

1. Do not wear loose clothing or open-toed shoes when sampling.
2. Wash hands before and after sampling.
3. Wear gloves at all times when sampling.
4. Be careful when sampling around operating equipment.
5. Be aware sponges may contain sensitive microbes and handle carefully at all times.

6. Never touch facial area when sampling.

Supplies:

Hydrasponge 5cm x 5cm sponges
Sterile paper towels
Sterile rubber gloves
Sterile whirlpak bags, 27 oz.
Sterile pipet, 10 ml

Reagents:

20% solution of bleach

Remarks:

1. Change gloves after sampling each site or before touching a new sampling sponge.
2. Do not open whirlpak bags until you are at the designated sampling site.
3. Keep whirlpak closed between sampling sites.

Procedure:

1. Preparation work should be done as close to the sample area as possible.
2. Disinfect setup area with 20% solution of bleach. Wipe clean with paper towels. Make sure to wear rubber gloves
3. Wash hands and put on sterile gloves. Be sure to avoid touching the external surface of the gloves with your hands.
4. Open packet and remove sponge.

Sampling

1. Environmental sponge sample should be conducted under floor mats, cracks in the wall, in areas of peeling paint, under tables, in crevices of equipment, drains, etc.
2. Hold the sponge aseptically with the sterile gloves and vigorously rub it over the area to be sampled.
3. A square meter (1 m²) may be sampled.
4. After swabbing, place sponge in a sterile whirlpak bag and seal.

5. A composite of up to 5 sponges from different locations can be taken and ran as one composite sample. If the sample results are positive, each site will be tested separately.
6. Record the following information on the whirlpak bag:
 1. Date
 2. Area Sampled
 3. Sampler's initials
7. Conduct all the above steps in preparation and sampling sections for all sponge samples taken.
8. Place whirlpaks in cooler with icepacks or fridge $\leq 8^{\circ}\text{C}$ for up to 24 hours if sample is not immediately plated.

Plating

1. After sample is obtained, dilute sponge with 25 ml of BFB media.
2. Squeeze sponge thoroughly to help flush any present microorganisms into BFB selective media.
3. Using 10 ml pipet, carefully transfer 1 ml of media sample onto appropriate EC/TC and or APC Petrifilm. Label Petrifilm with sample area description.
4. Transfer Petrifilm into 35°C incubator for 48 hours for further analyses.

References: "Compendium of Methods for the Microbiological Examination of Foods," 3rd ed., 1992

General Preparation of Media

Purpose: To ensure proper preparation of BFB and cultural media in the microbiological lab.

Safety Precautions:

1. Wear gloves and a lab coat at all times.
2. Use aseptic technique.
3. Do no open autoclave to remove media until pressure gauge or display has reached 0 psi.
4. Do not use cracked or chipped glassware.

Supplies:

Erlenmeyer Flask
Media Bottles
Test tubes
Plastic Bottles
Autoclave Tape
Marking Pen

pH meter
Autoclave, 121°C
Cultural media Kits
Glass pipettes
Degassing Flask
Vacuum Pump

Reagents:

Media
Distilled or Deionized Water
1N NaOH
1N HCL

Remarks:

1. Gloves should be worn at all times because media powders are very hygroscopic. Also, make sure that all lids are closed tightly after use.
2. Do not use powdered media that is beyond expiration date.
3. BFB media unlike cultural media can be made outside of a laminar flow hood.

Procedure:

BFB

1. For BFB media, check media container for directions on stock solution formula.
2. 60 ml of stock solution needs to be added to every 50 L of deionized water.
3. Once mixed with stock solution, if necessary, adjust the media to the proper pH using the pH meter. Use NaOH 1 N or HCL 1 N solution to adjust the pH.
4. Log in newly prepared media into a logbook in the following format:
(e.g. M071508A)
M = media
07 = month of the year
15 = day of the year
08 = the year
A = the preparation of the day (A represents the first preparation of any media on that day, B would be the second, etc.)

Cultural Media

1. Every cultural media used in the TE lab has a specific formula and SOP.
2. Always prepare media under laminar flow hood with airflow constantly on.
3. Use appropriate pipettes, degassing media flask, flask filter, and correct number of bottles as needed.
4. Do not prepare cultural media without correct working knowledge of aseptic techniques and at minimum one training session with experienced researcher.

References: Refer to the direction labels on the individual media kits or reference sheet in the TE lab and or media bottles.

Specifications, Sterilization & Cleaning of Laboratory Glassware and Plasticware

Purpose: To assure the quality and cleanliness of laboratory glassware and plasticware.

Safety Precautions:

1. To prevent cuts, always discard cracked or chipped glassware.

Procedure:

Specifications

1. Glassware should be of high quality, low alkali, borosilicate glass.
2. Plasticware should be free of defects and contain no toxic residues.
3. Glassware or plasticware with chipped or broken edges, or etched surfaces will be discarded.
4. Glassware and plasticware should be randomly checked on a bi-weekly basis for alkalinity and acidity by using bromothymol blue pH indicator. pH of glassware should be between 6.5 and 7.5.

General

1. Use hot water when cleaning laboratory glassware or plasticware.

2. Use approved laboratory detergent.
3. Wear gloves at all times when washing glassware or plasticware.
4. Clean all surfaces of the glassware and plasticware thoroughly using a brush.
5. Rinse glassware at least 8 times with running tap water followed by three rinses of distilled water to ensure¹⁹⁷ at all soap residues are removed.

Removal of “Milk Stone” and other Residue Deposits

1. Wash and rinse glassware following the general procedures.
2. Place glassware in an acid-dichromate cleaning solution for 24 hours. Avoid getting solution on self. Wear protective equipment at all times.
3. After 24 hours, remove glassware. Rinse thoroughly 12 times in running tap water and 3 times in distilled water.
4. After rinsing, test to see if all of the solution has been removed from the glassware. Place one drop of bromothymol blue pH indicator on the glassware. Glassware should have a pH between 6.5 – 7.5.
5. If acid is detected, rinse glassware several more times and repeat pH test.
6. If acid is not detected, rinse off bromothymol blue with distilled water and allow glassware to dry.

References: “Compendium of Methods for the Microbiological Examination of Foods,”
3rded., 1992

Handling of Media & Reagents

Purpose: To assure the quality of media and reagents used within the laboratory.

Safety Precautions: Always wear rubber gloves while handling media.

Supplies: N/A

Remarks:

1. All media will be used on a first in – first out basis. The oldest lot of media will be always be used first.
2. Any media that are older than the manufacturers’ expiration date, or do not pass quality control tests will be discarded immediately.

3. All media, reagents, bottles, tubes, plastic sleeves, etc. will be marked with the date of receipt, date of expiration and date of opening.

Procedure:

Receipt of Media and Reagents

1. Upon receipt, the manufacturer's name and number (lot, series, ID, etc.) will be verified to be the same on the invoice or packing list and the material's consumer.
2. The expiration date on the container shall be checked to ensure that expired or soon to be expired material has not been received.
3. The material's container shall be marked with the receipt date.
4. The received material shall be placed in the appropriate designated storage area.

Storage of Media

1. Store dehydrated media in the manufacturer's packaging, tightly capped bottles or tightly closed plastic liners in a cool, dry and dark area. If specified by the manufacturer, refrigerate the media.
2. Keep no more than six months to a year's supply on hand. Use older stocks first and do not use any media that has exceeded the manufacturers' expiration date.
3. Dehydrated media and reagents should be free floating powders or crystals. If a change is noted in this property or its color, the item in question should be discarded.
4. Media that contains dyes should be protected from light. Store them in a dark area, using a dark glass bottle or wrap the bottle in foil or brown paper.
5. All media prepared by the laboratory shall be stored in accordingly.
6. Each rack of prepared media shall be labeled with the type media, date prepared and the expiration date.
7. For pre-prepared media, consult package inserts for the manufacturer's specific shelf life or the shelf life will be three months from the date of manufacturer, whichever is shorter.

Opening of Media and Reagents

1. Upon opening of the media or reagent, the container shall immediately be marked with the date of opening and the expiration date may need to be

revised. The expiration date shall be either the actual manufacturer's expiration date or a date six months from the date of opening, whichever is earlier. If revised, the new expiration date shall be marked on the container.

2. Additionally, media shall be inspected for volume, tightness of closure, clarity, color, consistency and completeness of label. Any discrepancies shall be noted in the media/reagent control notebook.
3. Any media whose integrity is questionable due to color, clarity or fails testing shall be discarded immediately.

References: "Compendium of Methods for the Microbiological Examination of Foods," 3rded., 1992 Standard Methods for the Examination of Water and Wastewater, 18th Edition.

Cleanliness Testing of Researchers Hands & Gloves

Purpose: Provide for consistent sampling and analysis of hands and gloves for cleanliness using sponges as a measure of testing.

Safety Precautions: Use of sterile rubber gloves or sterile tongs is required to prevent cross contamination of personnel and samples.

Supplies:

1. Use (5cm x 5cm) sterile sponge placed in individual craft paper bags, or alternatively, commercially available, prehydrated sponges (5cm x 5cm)
2. Sterile whirl-pak bags are suitable to contain the sponges after sampling.
3. Sterile nutrient broth or 0.1% peptone water may be used as the rinse solution.
4. Sterile tongs or sterile gloves may be used to hold the sponge aseptically during sampling.

Remarks: N/A

Procedure:

1. Moisten the sponge (if non-hydrated) with 10 ml of sampling fluid (rinse solution).

2. While holding the sponge aseptically with tongs or sterile gloves swab the surface of the hand vigorously rubbing the sponge over hand area.
3. After sampling, place the sponge aseptically in a sterile bag and transport it to the laboratory under refrigeration.

Plating and Analysis

1. For quantitative analyses 50-100 ml of diluent is added to the bag containing the sponge. The sponge is vigorously massaged with diluent for 1-2 minutes to release the microorganisms. Aliquot of the diluent is removed from the bag and dilute further if required, and plated onto the desired petrifilm for the microorganisms. Number of microorganisms per unit surface can be calculated on the basis of the area swabbed (1m^2), and the amount of diluent used and size of aliquot plated.
2. For example:
 - *If 50 colonies are obtained from 1 ml aliquot derived from sponge in 100 ml of diluent that swabbed 1 m^2 (sq m) the count per m^2 will be:
 5000 cfu/m^2
Or 0.5 cfu/cm^2
 - *To compare effectively of rinse solution, the same method can be used for hands and gloves before and after dipping hands or hands with gloves into disinfecting rinse solution.

References: "Compendium of Methods for the Microbiological Examination of Foods," 3rd ed., 1992

Heterotrophic Plate Count/Pour Plate Method

Purpose: To estimate the number of live heterotrophic bacteria in water and measure changes during water treatment and distribution of water in cell culture uses.

Safety Precautions:

1. Wear rubber gloves appropriate to the hazards involved.
2. Dispose of all materials in the biohazard waste upon completion of this analysis.
3. Wash hands after performing this analysis or when leaving the microbiology lab.

Supplies:

Incubator, 35°C ± 0.5°C
 Sterile Petri Dishes 100 mm x 15 mm
 Sterile Gloves
 Sterile Pipets 1 ml
 Quebec Colony Counter

Remarks:

1. Do not use any prepared or dehydrated media after the expiration date.
2. Always run appropriate controls when conducting SPC analysis.
3. Check newly prepared lots of SPC agar for sterility before use.
4. After water samples have been collected initiate analysis as soon as possible.
5. Duplicate samples shall be done for all samples. They shall be run using Standard Plate Count Agar.
6. Control blanks for media, dilution water, plates, pipets and other materials shall be included per sample.
7. Control air blanks (sedimentation) shall be done at least monthly. Air blanks shall be exposed to the laboratory air for 15 minutes and then incubated per this SOP. The number of colonies shall not exceed 15 colonies/plate 15 minute exposure to be acceptable.

Procedure:

Preparation of Standard Plate Count Agar

Tryptone	5.0g
Yeast Extract	2.5g
Glucose	1.0g
Agar	15g
DI Water	1L

1. Mix appropriate amount of media in a liter of deionized water until evenly dispersed.
2. Heat with repeated stirring and boil for one minute to dissolve completely.
3. Place foil over the top of the container.

4. Autoclave for 15 minutes at 121°C, pH of SPC should be 7.0 ± 0.2 after autoclaving.
5. After autoclaving, remove container of agar and place into incubator at 44-46°C until needed for plating. SPC Agar may be stored up to 48 hours before use.
6. Do not re-sterilize plating medium.
7. Log the preparation of the agar into the media/reagent prep book. Include the following information:
 - a) Date
 - b) Type of media prepared
 - c) Preparer's Initials
 - d) Manufacturer's Name, Lot #, Expiration Date
 - e) Amount of agar used
 - f) Amount of agar prepared
 - g) Lab number
8. Log in the incubator logbook the time the agar is put into the incubator.

Plating the Samples

1. For most potable water samples, plates suitable for counting will be obtained by plating 0.1 ml of undiluted sample and 1 ml and 0.1 ml of 10^2 dilution.
2. All plating work must be done within the laminar flow hood.
3. Slowly pour the agar over the sample (Approximately 15-20 ml) and swirl gently.
4. Cover the plate immediately and allow agar to solidify.
5. Maintain remaining melted medium in water bath between 44-46°C until used.
6. Plates are now ready for incubation.

Incubation of SPC Agar Plates

1. Once the SPC plates have solidified, place them in the incubator at $35^\circ\text{C} \pm 0.5^\circ\text{C}$ for 48 hours.
2. Log the samples into the incubator logbook.
3. Plates may be removed after 24 hours for an initial colony count, but must be placed back in the incubator for remaining 24 hours.

Counting of SPC Plates

1. Turn plate upside down so you are looking at the bottom of the petri dish.
2. Count the number of colonies you see growing on the agar surface.
3. Counts should be recorded in CFU/plate.

4. Count plates should contain 30-300 colonies/plate. If the count are less than 30 or greater than 300, refer to the requirements of the *Standard Methods of 18th Edition for the Examination of Water and Wastewater*, Section 9215A.8

References: Standard Methods for the Examination of Water and Wastewater 18th Edition

Staph Count on Petrifilm

Purpose: To ensure proper use of the Petrifilm Staph Count system.

Safety Precautions:

1. Always wear gloves and lab coat when plating samples.
2. Wash hands after plating samples and handling Petrifilm.
3. Wash hands prior to leaving the laboratory area.

Supplies:

Incubator
1 ml Sterile Pipets
Sterile Buffer Tubes

Staph Express Count Plate
Staph Express Disk
Petrifilm Spreader

Reagents:

Sterile Butterfield's Buffer

Remarks:

1. A positive and negative control will only be necessary if *S. Aureus* or a sub species needs to be identified in the TE lab. Confirmations will not be performed at this junction.
2. Do not use plates and disks after expiration date.
Express Count Plates – storage requirements:
 - a. Store unopened pouches at 8°C or colder (allow pouch to come to room

- temperature before opening).
- b. Store resealed pouches in a cool dry place for no longer than 1 month.
Do Not Refrigerate
 - c. If the laboratory temperature exceeds 25°C (77°F) or the humidity exceeds 50%, then store the resealed pouches in a freezer.
3. Express Disk – storage requirements:
 - a. Store unopened pouches at 8°C or colder
 - b. As the disks are sensitive to moisture and light, remove only those disks that will be used immediately. Store resealed pouches in a sealable container in freezer for no longer than 6 months.
 4. Do not use plates or disks that show discoloration.

Procedure:

1. Prepare a 1:10 or greater dilution of media or swab tube. Weigh or pipette food into an appropriate container such as stomacher bag, dilution bottle, or other sterile container.
2. Add appropriate quantity of sterile Butterfield's phosphate-buffered dilution water.
3. Blend or homogenize sample per current procedure.
4. Adjust the pH of the sample to between 6.0 – 8.0. For acidic products adjust pH with 1 N NaOH. For alkaline products adjust pH with 1 N HCL.
5. Place Petrifilm plate on level surface. Lift top film. With the pipet perpendicular to petrifilm plate, place 1 ml of sample onto center of the plate.
6. Carefully roll top film down to avoid entrapping air bubbles. Do not let top film drop.
7. Gently apply pressure on spreader to distribute inoculums over circular area before gel forms. Lift spreader; do not twist or slide spreader. Wait a minimum of one minute for gel to solidify.
Note: Spread the sample on each individual plate before inoculating the next. This is important as the gel forms quickly and you may not be able to spread the sample if you inoculate more than 1 plate at a time.
8. Remove the spreader and leave the plates undisturbed for at least one minute to permit the gel to form.
9. Incubate plates in a horizontal position with the clear side up in stacks of no more than 10 plates. Incubate the plates for 24 ± 2 hours at $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$, or $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
10. Count the Staph Express plates with a standard colony counter or other illuminated magnifier. Do not count colonies which appear on the foam dam because they are removed from the selective influence of the medium.

11. Observe the colony colors:
 - a. If no colonies or only red-violet colonies are present after 24 ± 2 hours, count red-violet colonies as *S. Aureus*, the test is complete. Use of the Petrifilm Staph Express disk is not necessary.
 - b. If any colony colors besides the red-violet are present, use a Petrifilm Staph Express disk.
12. Remove an individually packaged Petrifilm Staph Express disk from its pouch and allow it to come to room temperature. Then remove the disk from its individual package by peeling the package to expose the disk tab, grasping the tab, and pulling the disk out.
13. Lift the top film and place the disk in the well of the plate so that the tab remains outside the well.

Note: Occasionally the gel may split when the top film is lifted. Performance of the Petrifilm Staph Express plate is not affected by gel splitting because the disk is coated on both sides.
14. Lower the top film.
15. Apply pressure by sliding a gloved finger firmly across the entire disk area (including edges) to ensure uniform contact of the disk with the gel and to eliminate any air bubbles.
 - a. If too much pressure is applied, the gel may be disturbed, and zones may not be distinct.
 - b. If too little pressure is applied, the gel may dry, and zones may not form.
16. Place the disked Petrifilm Staph Express plates in a stack of more than 10 plates and incubate at $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$ or $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for at least 60 minutes and no longer than 3 hours. Check the plates periodically; the Dnase reaction may give final results in less than 3 hours.
17. Remove plates from incubator.
18. Count all pink zones whether or not colonies are present. Pink zones are usually associated with *S. aureus* but may indicate *S. hyicus*, or *S. intermedius*. Colonies not associated with pink zones are not *S. Aureus* and should not be counted. If the entire disked area is pink with no distinct zones, large numbers of *S. aureus* are present. Record the results as too numerous to count and dilute the sample further to obtain a more accurate count.
19. Isolate colonies for further identification, if necessary.

References: 3M Petrifilm Staph Express Count System insert

Standard Plate Count

Purpose: To assure proper plating, incubation and interpretation of the Standard Plate Count using the pour plate method.

Safety Precautions:

1. Always wash hands thoroughly before and after performing microbiological procedures and when leaving the laboratory.
2. Wear gloves appropriate to the hazards involved.
3. Always follow aseptic techniques.
4. Dispose of all hazardous materials in the biohazard waste upon completion of the analysis.

Supplies:

Incubator 35°C ± 0.5°C	Rubber Gloves
Incubator 44-46°C ± 0.5°C	Sterile Pipets
Sterile Petri Dishes	Quebec Colony Counter

Media: Standard Plate Count Agar (SPC) or Plate Count Agar (PCA), 44-46°C

Remarks:

1. Do not use any prepared media after the expiration date.
2. Always run the appropriate blank control when conducting SPC analysis.
3. SPC is also known as Total Plate Count (TPC), Heterotrophic Plate Count (HPC) or Aerobic Plate Count (APC).
4. Check newly prepared lots of SPC agar for sterility before use.
5. This method can be used for analysis of product, water, media, and laboratory sedimentation plate samples.
6. For each series of water samples being tested for HPC, duplicate sample tests shall be run.
7. Do not test any water with an elapsed time greater than 30 hours between sampling and commencement of testing.
8. Controls should be run for each series of samples – media, sterility, dilution, blanks, air and pipet.

9. Remove media from incubator and allow it to cool slightly just prior to pouring the plates.
10. All plating work, with the exception of laboratory sedimentation plates, must be done within a laminar flow hood.

Procedure:

Plating of Media Samples

1. Prepare your samples according to the "Microbiology Sampling Preparation."
2. Place 1.0 mL or 0.1 mL of sample or dilution into a sterile Petri dish and cover.
3. Repeat step 2 for all dilutions that need to be plated.
4. Remove the SPC agar from the incubator or warm water bath and allow to cool for 10-15 minutes.
5. Slowly pour the SPC agar into the Petri dishes containing the sample (approximately 15-20 mL). Cover the plate immediately.
6. Swirl the Petri dishes gently in a figure 8 motion to ensure that the sample is distributed evenly in the agar.
7. Allow the agar to solidify before turning the plate over for incubation.

Plating of Water Samples for HPC

1. For most potable water samples, plates suitable for counting will be obtained by plating 1.0 mL (10^0 dilution) and 0.1 mL (10^{-1}) of the undiluted sample.
2. Place 1.0 mL and/or 0.1 mL of sample into a sterile Petri dish and cover.
3. Slowly pour the SPC agar into the Petri dishes containing the samples (approximately 15-20 mL). Cover the plate immediately.
4. Swirl the Petri dishes gently in a figure 8 motion to ensure that the sample is distributed evenly in the agar.
5. Allow the agar to solidify before turning the plate over for incubation.

Laboratory Sedimentation Plates

1. Slowly pour approximately 15-20 mL of SPC agar into the Petri dish.

2. Let the Petri dish stand uncovered and undisturbed for 15 minutes on the laboratory counter top, (the test can also be performed in the TE lab room).
3. After 15 minutes has elapsed, cover the plate and incubate as follows.

Incubation of SPC Agar Plates

1. Once the SPC plates have solidified, invert the plates and place them into the incubator at 35°C for 48 ± 3 hours. Do not stack plates greater than 4 high.
2. During incubation, maintain humidity in the incubator so the plates lose no more than 15% moisture.
3. Plates may be removed after 24 hours for an initial colony count; then they must be placed back into the incubator for the remaining 24 hours.

Counting of SPC Plates

1. View the plate through the bottom of the Petri dish.
2. Count the number of colonies you see growing on the agar surface.
3. All counts shall be recorded as CFU/ml and CFU/Plate/15 mins (lab sedimentation). Be sure to account for any dilutions that were made in the sample when recording counts on the appropriate paperwork.
4. All counts not between 25-250 (media) and 30-300 (water) need to be recorded as estimates.
5. For any plates that are higher than noted above, average the number of colonies in one square (1 cm²) of the Quebec Colony Counter and multiply by 50 to obtain the total count per plate.

References: Compendium of Methods for the Microbiological Examination of Foods, 4th Ed. Standard Methods for the Examination of Water and Wastewater, 20th Ed.

Appendix I - *Media recipes*

Endothelial Cell Media (*EC Media kit from Lonza: Stored at -20° C*)

- 10 ml Fetal Bovine Serum (FBS)
- 0.2 ml Hydrocortisone
- 2 ml hFGF- β
- 0.5 ml VEGF
- 0.5 ml R3-IGF-1
- 0.5 ml Ascorbic Acid
- 0.5 ml hEGF
- 0.5 ml GA-1000
- 0.5 ml Heparin
- 1 bottle M199 (500 ml)

Bioreactor Media

- 1 bottle M199 (500 ml)
- 56 ml FBS
- ml L-glutamine
- ml Pencilin/Streptomycin (P/C)
- 0.6 ml Fungizone
- 2.8 ml Hepes buffer (clear solution)

Appendix J – Microtome Protocol

1. Fill warm water pot with distilled water and allow warming up.
2. Put embedded wax blocks of vessel cross section in fridge for 20 minutes between 3 - 5°C prior to cutting.
3. Use microtome handle and engage brake system when slicer is not being used.
4. Set slicer setting to 7 µm to obtain sections.
5. If sections decompose or wither set slicer to higher settings to obtain uniform and structured slices.
6. Slowly work the slicer back down to thinner cross sections to obtain desired cutting size for histology.
7. Preserve as much as the sample as possible for future use if needed.
8. After slices have been obtained, use forceps to transfer wax slices into the warm water bath.
9. Transfer slice onto to the rough part of the staining slide.
10. Caution: Never let mandrel come out to far, if uncertain of this ask an appropriate supervisor or trained individual.
11. Navigate sample onto rough side of the staining slide with forceps from warm water bath.
12. Use brushes to thoroughly clean all equipment.
13. Transfer slide to slide case for histology staining.

Appendix K – HUVEC Density Values for BVM Studies

Study One

Vessel A	3.0×10^6	(Cells/cm ²)
Vessel B	3.0×10^6	(Cells/cm ²)
Vessel C	3.4×10^6	(Cells/cm ²)
Vessel D	3.4×10^6	(Cells/cm ²)
Vessel E	4.0×10^6	(Cells/cm ²)
Vessel F	4.0×10^6	(Cells/cm ²)

The resuspension density for the vessels was found by dividing the expected cell count times the confluency observed by the amount of EC media used to resuspend the pellet; in study one 5 ml of EC media was used per pellet.

Vessel A	1.4×10^6	(Cells/cm ²)
Vessel B	1.6×10^6	(Cells/cm ²)
Vessel C	1.6×10^6	(Cells/cm ²)
Vessel D	1.6×10^6	(Cells/cm ²)
Vessel E	2.0×10^6	(Cells/cm ²)
Vessel F	2.0×10^6	(Cells/cm ²)

Study Two

Vessel 1	2.5×10^6	(Cells/cm ²)
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The BVM had a final cell resuspension density of 8.3×10^5 cells/ml. The amount of cells was multiplied by the observed confluency and divided by exposed the surface area of the lumen (2.36 cm²).

Study Three

Vessel 1	3.0×10^6	(Cells/cm ²)
Vessel 2	3.0×10^6	(Cells/cm ²)
Vessel 3	3.4×10^6	(Cells/cm ²)

Vessel 4	3.4×10^6	(Cells/cm ²)
Vessel 1	2×10^6	(Cells/ml)
Vessel 2	2×10^6	(Cells/ml)
Vessel 3	3×10^6	(Cells/ml)
Vessel 4	3×10^6	(Cells/ml)

BVMs 1 and 2 had final cell resuspension densities of 2.0×10^6 cells/ ml. For BVMs 3 and 4, the vessels were seeded with a density of 3 cells/ cm² with a resuspension density value for the amount of cells introduced into the vessel at 3×10^6 cells/ml; based on the amount of EC media used to resuspend the HUVECs.