



Engineering a Biomimetic Adventitia to Model Fibrosis in a Tissue-Engineered Blood Vessel

Citation

Kepler, Sydney Christina. 2023. Engineering a Biomimetic Adventitia to Model Fibrosis in a Tissue-Engineered Blood Vessel. Bachelor's thesis, Harvard University Engineering and Applied Sciences.

Link

<https://nrs.harvard.edu/URN-3:HUL.INSTREPOS:37378290>

Terms of use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material (LAA), as set forth at

<https://harvardwiki.atlassian.net/wiki/external/NGY5NDE4ZjgzNTc5NDQzMGIzZWZhMGFIOWI2M2EwYTg>

Accessibility

<https://accessibility.huit.harvard.edu/digital-accessibility-policy>

Share Your Story

The Harvard community has made this article openly available.

Please share how this access benefits you. [Submit a story](#)

Engineering a Biomimetic Adventitia to Model Fibrosis in a Tissue-Engineered Blood Vessel

A senior design project report submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Engineering Sciences (Bioengineering) at Harvard University

Sydney Christina Kepler

S.B. Degree Candidate in Engineering Sciences (Bioengineering)

Faculty Advisor: Dr. Elliot Chaikof

Advisor: Dr. Richard Cheng

Thesis Reader: Dr. Jennifer Lewis

Harvard John A. Paulson School of Engineering and Applied Sciences

Cambridge, MA

March 31, 2023

Table of Contents

TABLE OF CONTENTS	2
LIST OF FIGURES	4
LIST OF TABLES	4
LIST OF EQUATIONS.....	4
ABBREVIATIONS	5
ACKNOWLEDGEMENTS	6
ABSTRACT.....	7
1. INTRODUCTION.....	8
1.1. BACKGROUND AND MOTIVATION.....	8
1.2. PROBLEM STATEMENT	9
1.3. PROJECT GOALS & END USERS	10
1.4. TECHNICAL CHALLENGE	10
1.5. CONTEXT AND ETHICAL CONSIDERATIONS.....	11
2. BACKGROUND RESEARCH.....	13
2.1. TISSUE-ENGINEERED BLOOD VESSEL FABRICATION	13
2.2. ADVENTITIA FABRICATION	14
2.3. IN VITRO FIBROSIS MODELS.....	15
2.4. PREVIOUS WORK	16
3. DESIGN REQUIREMENTS & APPROACH	18
3.1. DESIGN-INDEPENDENT SPECIFICATIONS	18
3.2. DESIGN-DEPENDENT SPECIFICATIONS.....	19
3.3. DESIGN APPROACH.....	21
3.4. SYSTEM DIAGRAM	31
4. MATERIALS & METHODS FOR FABRICATION.....	35
4.1. MATERIALS PREPARATION	35
4.1.1. CELL CULTURE	35
4.1.2. COLLAGEN SOLUTION & BUFFER PREPARATION	35
4.1.3. MEDIAL & INTIMAL TEBV FABRICATION	36
4.2. ADVENTITIA ENGINEERING.....	36
4.3. METHODS OF ADVENTITIA CHARACTERIZATION	37
4.3.1. CELL VIABILITY & PROLIFERATION	37
4.3.2. LAYER RADIAL THICKNESS	37
4.3.3. RT-PCR GENE EXPRESSION ANALYSIS.....	38
5. EVALUATION OF RESULTS.....	41
5.1. CELL VIABILITY ANALYSIS.....	41

5.2.	LAYER THICKNESS.....	42
5.3.	GENE EXPRESSION ANALYSIS	44
6.	DISCUSSION	49
6.1.	ACHIEVEMENT OF TECHNICAL SPECIFICATIONS.....	49
6.2.	COMPARISON TO PREVIOUS MODELS	51
6.3.	PROJECT LIMITATIONS	52
6.4.	FUTURE WORK: MODELING FIBROSIS.....	57
6.4.1.	MODELING AND CHARACTERIZING FIBROSIS.....	57
6.4.2.	PRELIMINARY WORK: SORTING FIBROBLASTS, SMOOTH MUSCLE CELLS, & MYOFIBROBLASTS.....	58
6.5.	PROJECT IMPACT	62
	REFERENCES.....	63
7.	APPENDIX.....	66
7.1.	FACS & RT-PCR DATA.....	66
7.2.	MODELING FIBROSIS	70
7.3.	ENGINEERING STANDARDS.....	75
7.3.1.	ASTM F3504-21	75
7.3.2.	ASTM F3510-21	76
7.3.3.	ASTM F2603-06	76
7.4.	BUDGET.....	77

List of Figures

FIGURE 1: SCHEMATIC DEPICTING THE THREE MAIN LAYERS OF AN ARTERY: THE TUNICA INTIMA, TUNICA MEDIA, AND TUNICA ADVENTITIA.....	8
FIGURE 2: SCHEMATIC DEPICTING MOLDING METHOD.....	14
FIGURE 3: SCHEMATIC DEPICTING THE ROLLING TEBV FABRICATION METHOD.....	14
FIGURE 4: EVALUATION OF FIBROBLAST GROWTH OVER 3 DAYS AT INCREASING CELL DENSITIES.....	25
FIGURE 5: THE R_1 AND R_0 OF A TRI-LAYERED TEBV.....	27
FIGURE 6: MTS ASSAY STANDARD CURVE.....	30
FIGURE 7: SCHEMATIC OF TEBV FABRICATION, PHASE 1 OF THE DESIGN PROCESS.	32
FIGURE 8: ADVENTITIAL ENGINEERING, PHASE 2 OF THE DESIGN PROCESS.....	33
FIGURE 9: MODELING FIBROSIS, PHASE 3 OF THE DESIGN PROCESS.....	34
FIGURE 10: SSC VS. FSC DENSITY PLOTS FROM FACS ANALYSIS.	39
FIGURE 11: CELL VIABILITY OF TEBVS CONTAINING THE MEDIAL AND ADVENTITIAL LAYERS.....	42
FIGURE 12: EVALUATION OF ADVENTITIAL LAYER THICKNESS IN TEBV CONSTRUCTS.	43
FIGURE 13: EVALUATION OF FLUORESCENCE-ACTIVATED CELL SORTING (FACS) RETRIEVAL OF FIBROBLAST AND SMOOTH MUSCLE CELL POPULATIONS.	46
FIGURE 14: RT-PCR ANALYSIS OF SMOOTH MUSCLE CELL SAMPLES DIGESTED FROM TEBVS INCLUDING AN ADVENTITIAL LAYER.....	48
FIGURE 15: SETUP OF TISSUE-ENGINEERED BLOOD VESSEL (TEBV) ROLLING PROCESS LEADING TO CONTAMINATION.....	54
FIGURE 16: STITCHED CROSS-SECTIONAL IMAGE OF A TEBV.....	56
FIGURE 17: EXPERIMENTAL SETUP OF TGF-B EXPOSURE TO SMCs AND FBs.	59
FIGURE 18: CD 90 & CD140b FACS HISTOGRAMS OF FB SAMPLES WITH AND WITHOUT TGF-B EXPOSURE.	61
FIGURE 19: GATING STRATEGY FOR FB & SMC FACS.	67
FIGURE 20: RPLP0 AMPLIFICATION CURVE.....	68
FIGURE 21: COL1A1 AMPLIFICATION CURVE.....	68
FIGURE 22: ACTA2 AMPLIFICATION CURVE..	69
FIGURE 23: MYH11 AMPLIFICATION CURVE..	69
FIGURE 24: GATING STRATEGY FOR SMC, FB, AND MYOFB SORTING.	72
FIGURE 25: CELL DENSITY PLOTS FOR TGF-B ⁻ (LEFT) AND TGF-B ⁺ (RIGHT) SMC SAMPLES.	73
FIGURE 26: CELL DENSITY PLOTS FOR TGF-B ⁻ (LEFT) AND TGF-B ⁺ (RIGHT) FB SAMPLES.	74
FIGURE 27: CELL DENSITY PLOTS FOR TGF-B ⁻ (LEFT) AND TGF-B ⁺ (RIGHT) CO-CULTURE SAMPLES.	75

List of Tables

TABLE 1: MERITS & PITFALLS OF EXISTING FIBROSIS MODELS.	15
TABLE 2: ROLLING VS. MOLDING METHODS FOR TEBV FABRICATION.	17
TABLE 3: DESIGN-INDEPENDENT TECHNICAL SPECIFICATIONS & MEASUREMENT METHODS.	18
TABLE 4: DESIGN-DEPENDENT TECHNICAL SPECIFICATIONS & MEASUREMENT METHODS.....	20
TABLE 5: DESIGN-DEPENDENT TECHNICAL SPECIFICATION PUGH MATRIX FOR DESIGN APPROACHES.....	22
TABLE 6: NUMBER OF FIBROBLASTS (FBs) AND SMOOTH MUSCLE CELLS (SMCs) SEEDING PER WELL.....	29
TABLE 7: ANALYSIS OF DESIGN SPECIFICATION ACHIEVEMENT.....	49
TABLE 8: DATA FOR CELL YIELD BEFORE AND AFTER FACS.	66
TABLE 9: ISOLATED RNA CONCENTRATIONS AND PURITY RATIOS FOR FACS SAMPLES.	66
TABLE 10: POTENTIAL DESIGN-INDEPENDENT TECHNICAL SPECIFICATIONS & MEASUREMENT METHODS FOR MODELING FIBROSIS.	70
TABLE 11: UPDATED ITEMIZED BUDGET.	77

List of Equations

EQUATION 1: ADVENTITIAL SHEET LENGTH CALCULATION WITH E* ERROR FACTOR.	26
EQUATION 2: PERCENT CELL VIABILITY CALCULATION USING KNOWN SEEDING DENSITY AND MEASURED CELL DENSITY FROM THE GENERATED STANDARD CURVE.	31

Abbreviations

Cardiovascular disease (CVD), Tissue-engineered blood vessel (TEBV), extracellular matrix (ECM), smooth muscle cell (SMC), fibroblast (FB), endothelial cell (EC), myofibroblast (MyoFB), transforming growth factor-beta (TGF- β), (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS), Fluorescence-Activated Cell Sorting (FACS), Real-Time Polymerase Chain Reaction (RT-PCR), Beth Israel Deaconess Medical Center (BIDMC)

Acknowledgements

This project would not have been possible without the support of several people. First, I would like to thank my faculty advisor Dr. Elliot Chaikof for allowing me the opportunity to work in his laboratory. Additionally, I would like to thank Dr. Richard Cheng and Dr. Torsten Meissner for their invaluable guidance and mentorship over the past two years. Their support has been integral to my development as a researcher and as a person.

Next, thank you to the many people who provided assistance and advice for my project. First, thank you to Dr. Jennifer Lewis for graciously agreeing to serve as my thesis reader and giving me invaluable feedback through the year. Thank you to Tom Ferrante at the Wyss Institute for taking the time to train me to use the multi-photon microscope. Thank you to Garrett Haskett, John Tigges, and Tim Peach at the BIDMC Flow Cytometry Core for their support with FACS instrument booking, usage, and data processing. Thank you to Alex Lovely at the Harvard Center for Biological Imaging for providing me with trainings and his support with confocal microscopy.

Next, I would like to thank the Active Learning Labs and ES 100 staff, especially Dr. Linsey Moyer, Dr. Melissa Hancock, and Avery Normandin, each of whom provided important feedback and support during the progression of this project.

Finally, I would like to thank the countless friends and family members whose support and listening ear helped to guide me through what initially seemed to be an impossible task. I would especially like to thank my fellow engineering peers Adam McMullin, Catherine Mailly, Bianca Byfield, Jaxson Hill, and Jason Martel for their friendship, empathy, and support over the past four years.

Abstract

Cardiovascular disease (CVD) is the global leading cause of death. CVD is commonly caused by fibrosis, or the stiffening of arterial vessel walls. While arterial fibrosis has several pathophysiologies, the outermost vessel layer, the tunica adventitia, has gained interest as a critical player in arterial fibrosis.

Due to the simplicity and lack of tunability of existing fibrosis models, it remains difficult to understand the significance of the adventitia in fibrosis development. Thus, there exists a need to create an *in vitro* model of the adventitia to better understand its role in the development of vascular fibrosis.

Towards this end, this project includes working in the Chaikof Lab to optimize the fabrication of the adventitial layer itself and its incorporation into the current TEBV model. On a larger scale, the goals of this project are to use a tri-layered TEBV to study arterial fibrosis *in vitro* and screen anti-fibrotic drug candidates.

1. Introduction

1.1. Background and Motivation

Cardiovascular disease (CVD) is the leading cause of death in the US and globally [1]. CVD is commonly caused by fibrosis, or the stiffening of arterial vessel walls. In early-stage CVD, activated cells around arteries secrete excess amounts of collagen and stiffen the vessel walls. These fibrotic vessels can lead to further complications, such as plaque buildup leading to atherosclerosis and coronary heart disease [2].

The complex components of arterial anatomy play key roles in the development of fibrosis and, ultimately, CVD. Arteries have three main layers: the tunica intima, the tunica media, and the tunica adventitia.

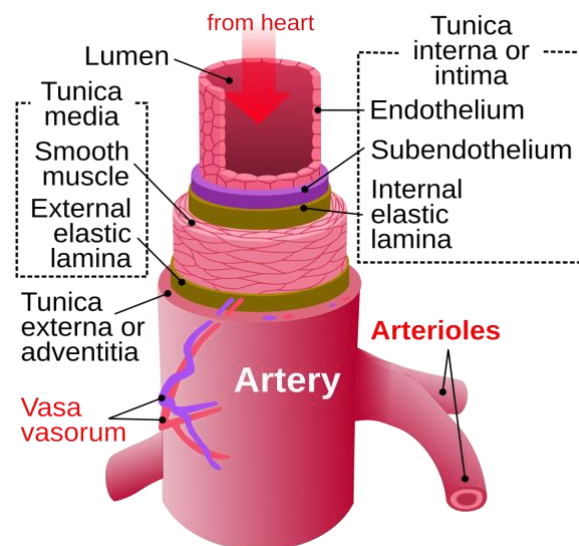


Figure 1: Schematic depicting the three main layers of an artery: the tunica intima, tunica media, and tunica adventitia. Adapted from [5].

The innermost layer of an artery is referred to as the tunica intima. The tunica intima consists of a monolayer of endothelial cells (ECs) and supporting layers of collagen fibers, and it is the layer that directly contacts blood flowing through the vessel. The tunica media is made up

of smooth muscle cells (SMCs), connective tissue, and collagen fibers. Finally, the outermost layer is the tunica adventitia, which is composed of collagen and elastin, as well as several different cell types. The most abundant cell type found in the adventitia are fibroblasts (FBs), which help to provide structural support to the artery [3].

When an artery experiences an injury or disease, SMCs in the medial layer proliferate, and some transition into myofibroblasts (MyoFBs)[4]. In the adventitial layer, quiescent FBs transition into active MyoFBs. Upon MyoFB activation, they both secrete ECM around the injury site and are responsible for contracting the deposited collagen around the wound, both important phenomena for wound healing [5]. In diseases such as arterial fibrosis, however, MyoFBs remain activated and continuously deposit ECM that stiffens the arterial walls, leading to further complications such as CVD.

Various pathways have been identified as promoting FB transition to active MyoFBs. It has been well-documented that several growth factors, most notably Transforming Growth Factor-Beta (TGF- β), promote MyoFB activation [6]. Along with chemical stimuli, it has been noted that mechanical stimuli, such as increasing stress and strain on the tissue, are also important in MyoFB activation and fibrosis formation. Activated MyoFBs release signaling factors, such as TGF- β 1, that promote even further MyoFB transition, creating a fibrotic positive feedback loop.

1.2. Problem Statement

When MyoFBs present in the adventitia remain activated, they continuously deposit collagen fibrils that contribute to arterial stiffening and the progression of cardiovascular diseases. Due to the simplicity and lack of tunability of existing fibrosis models, it remains difficult to understand the significance of adventitial FBs in fibrosis development in a lab-based

platform [7]. Thus, there exists a need to create an *in vitro* model of the adventitia to better understand its role in the development of vascular diseases such as fibrosis.

1.3. Project Goals & End Users

Towards this end, the goals of this project included working in the Chaikof Lab to develop a tissue-engineered blood vessel (TEBV) that can be used for disease modeling and animal engraftment. The Chaikof Lab has established a fabrication process to develop a TEBV consisting of the two innermost vessel layers, and this project works to add to this model's complexity with the addition of the tunica adventitia. On a larger scale, the end-users of this project will be a company or research lab interested in investigating potential anti-fibrotic therapies. The ultimate motivation of this project was to aid researchers in better understanding the pathophysiology of CVD and to develop a tool for preliminary drug screening for reversing fibrosis.

1.4. Technical Challenge

The purpose of this project was to develop an *in vitro* fibrotic TEBV that can be used to better understand early-stage cardiovascular disease (CVD) and for screening new drug candidates to treat fibrosis. This project, involving the optimization of the development and incorporation of the adventitial layer to the fabricated two-layered TEBV, was technically challenging to engineer a vessel layer that can be integrated into the medial and intimal TEBV layers. In addition, using the fibroblasts (FBs) embedded within the adventitial layer to model fibrosis will be a challenge, as there is not an established method for inducing fibrosis in an *in vitro* blood vessel. As this project progressed, each step was more technically challenging since it relied on the optimization and proper functionality of the previous work done as a whole. To

successfully complete this project, it was crucial to first become comfortable with the technically difficult process of rolling collagen sheets into TEBVs. Then, as outlined in more detail in **Sections 3, 4, and 5** the project proceeded with designing, building, and testing various parameters important to successfully engineering the arterial adventitial layer. Looking forward, various methods will be explored for using the fabricated adventitia to create an arterial model of fibrosis. Each of these processes involved using skills acquired through previous experiences with wet lab research, and it required the acquisition of new methods and techniques for data collection and analysis.

1.5. Context and Ethical Considerations

Context & ethical considerations were taken into account for the specification of this design project. From a public health lens, this project worked to advance the overall health of the millions of people living with cardiovascular disease. From a global lens, this fibrosis platform was difficult to produce on a scale widely available to those unaffiliated with a company or research lab in the client space. Many of the components used to construct this platform are biohazards and dangerous to use outside of a controlled wet-lab setting. In addition, it may be difficult and expensive to acquire some of the materials in certain countries. Nevertheless, the drug candidates potentially tested with this platform and deemed successful in treating cardiovascular diseases would ideally be readily available on a global scale. From a cultural lens, it could be considered nonideal for *in vivo* mouse models to be studied and sacrificed to better understand key components of cardiovascular disease and for drug screening purposes. In this context, a tissue-engineered fibrotic vessel model could be more ethically sufficient. In addition, it was important to take into account the ethical considerations of the cell sources used for this

project (human primary cells, induced pluripotent stem cells, embryonic stem cells, etc.). From a social lens, it was important to consider the implications of how better understanding the pathophysiology of cardiovascular disease and drug candidate screening could overshadow the societal factors that influence the disease, such as dietary and lifestyle factors. From an environmental lens, the proper disposal of chemicals and biological components used in the design process was considered. Minimizing the environmental impact of this work would maximize the impact of the project. From an economic lens, this project used cell sources that were expensive (over \$700 per vial). The data collection of this project, which included RT-PCR, flow cytometry, immunostaining, and multi-photon imaging, was economically taxing. It was important to narrow down only the required types of data collection to satisfy the project's technical specifications in an effort to minimize wasted time, resources, and funding.

2. Background Research

2.1. Tissue-Engineered Blood Vessel Fabrication

Several methods exist to manufacture a scaffold-free tissue-engineered blood vessel (TEBV). Two different fabrication approaches have previously been explored in the Chaikof Lab:

Molding TEBV Fabrication Method

Zhang et al. developed a popular method of TEBV fabrication that involves suspending cells in a collagen solution and molding the solution into a cylindrical shape around a central rod to create a blood vessel conduit [8]. The molded TEBV is removed from the mold and compressed by wicking away its water content to compact the collagen fibrils (**Figure 2**). This method has been widely adopted for *in vitro* TEBV fabrication towards further biological research. However, this fabrication process mixes smooth muscle cells (SMCs) and fibroblasts (FBs) together in a collagen solution, each at similar concentrations to their typical concentrations in their respective layers. This fabrication method fails to capture the complexity of separating the adventitial and medial layers.

While the structural advantages of the tunica adventitia are widely accepted, only more recently have the functional aspects of the adventitia become a source of interest to scientists. Moreover, it is hypothesized that the FBs within the adventitia have a key role in vascular injury response and fibrosis. With the current best models to study the role of the adventitia in cardiovascular disease being *in vivo* mouse and pig models, it is difficult to accurately isolate the role of FBs in the adventitia due to the *in vivo* complexity [7].

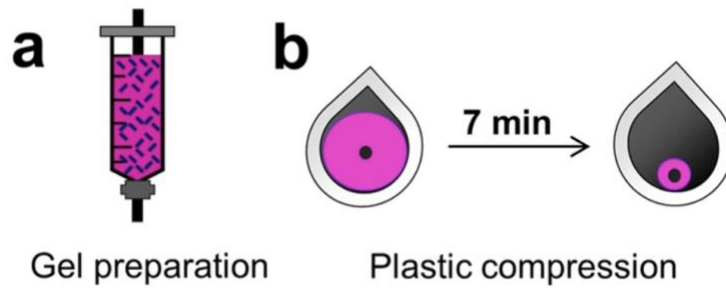


Figure 2: Schematic depicting molding method. (a) A collagen solution containing cells is molded in a syringe with a rod inserted in the middle (side view). (b) After gelation, the molded TEBV is removed and compressed to increase mechanical strength (cross-section). Adapted from [8].

Rolling TEBV Fabrication Method

The Chaikof Lab has developed a multi-step TEBV fabrication process for the tunica intima and tunica media. Three ultrathin collagen sheets are extruded and layered onto a glass slide, and smooth muscle cells (SMCs) are seeded and cultured on top of the layered slide for 3 days [9]. The SMC-laden sheets are rolled around a mandrel into vessel conduits. The TEBVs are endothelialized in a rotating plate to ensure a confluent monolayer of endothelial cells to create the tunica intima. This is the standard fabrication process for the base TEBV, and the adventitia was added to the conduit [10].

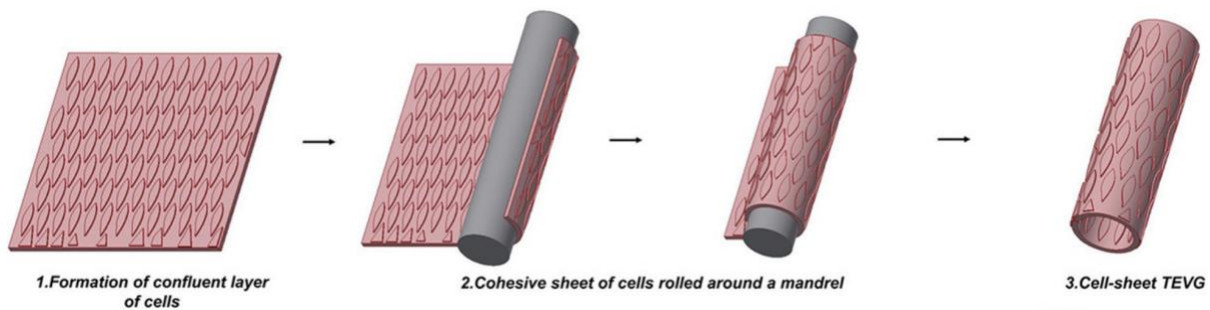


Figure 3: Schematic depicting the rolling TEBV fabrication method. Adapted from [10].

2.2. Adventitia Fabrication

Within the tissue engineering field, the distinct adventitial layer is often lost due to a co-culture of smooth muscle cells (SMCs) and fibroblasts (FBs), combining the adventitia and tunica media into one layer. For example, Zhang et al. designed a model of atherosclerosis using a TEBV fabricated by molding collagen embedded with SMCs and FBs into a tubular mold [8]. While this method allows for rapid model fabrication, it fails to capture the layered complexity of native blood vessel tissues.

There have not been significant efforts to engineer the distinct adventitial layer in TEBVs. Vascular tissue engineering efforts have been focused on the tunica intima and tunica media, even though the tunica adventitia is crucial for structural support and injury response. More recently, there have been few efforts to engineer the adventitial layer separately to provide extra mechanical support to TEBVs. Patel et al. used a ring formation and stacking method to fabricate adventitial blood vessels. They found that when embedded in a collagen solution, FBs deposited collagen fibrils that significantly helped to increase the mechanical strength of their vascular walls [11].

2.3. *In Vitro* Fibrosis Models

Several types of *in vitro* models have been used to study fibrosis. Below is a table including some of the most notable methods, their merits, and their pitfalls:

Table 1: Merits & Pitfalls of Existing Fibrosis Models.

Model Type	Merits	Pitfalls
2D mono/coculture [12] [13]	High throughput, simple bulk RNA sequencing & imaging, ease of model fabrication & handling	Lack of <i>in vivo</i> complexity, inability for <i>in vivo</i> translation

3D self-assembled models (spheroids & organoids) [14] [15]	Self-assembled organization (ease of fabrication), 3D cell-cell & cell-ECM interactions	Simplified models, low-throughput, low reproducibility, avascular
3D bio-printed models [16] [17] [18]	Precise tunability, high reproducibility of complex biomaterial & cell combinations	Low throughput, avascular, low structural integrity
Microphysiological systems (MPSs) & “organ-on-chip” [19] [20] [21]	Better capture <i>in vivo</i> environment, high throughput, can be combined with various organ systems	Avascular, simplified models

While existing *in vitro* models have been important steps toward developing representative fibrotic models, they each have their limitations. There exists a need for a vascular fibrotic model, as many of the existing models are avascular. In addition, employing tissue-engineering fabrication methods listed above could better allow for a complex *in vitro* model that can be more easily translated into *in vivo* conditions.

2.4. Previous Work

TEBV Fabrication: Rolling vs. Molding Methods

The Chaikof Lab has explored both methods of TEBV fabrication, and they have concluded that the rolling fabrication method results in a blood vessel conduit that more accurately represents native vessel tissue from a mechanical, biochemical, functional, and morphological lens, despite the time saved using a molding fabrication method.

Table 2: Rolling vs. Molding Methods for TEBV Fabrication.

Rolling TEBV Fabrication Method	Molding TEBV Fabrication Method
Collagen fibril and SMC alignment reflect physiological conditions	Collagen fibrils are unaligned and randomly aligned
Increased cell viability	Decreased cell viability
Increased mechanical strength (>1600 mmHg burst pressure)	Decreased mechanical strength (80 mmHg burst pressure)
Long, technically difficult fabrication (1 week)	Short, simple fabrication (1 hour)

3. Design Requirements & Approach

The goal is to develop an *in vitro* model of fibrosis using a tissue-engineered blood vessel (TEBV). The scope of this project focused on engineering and characterizing the addition of the adventitial layer to a two-layered TEBV. Towards this end, the design-independent technical specifications address the characterization of a tri-layered TEBV. The proposed design approach is to engineer an adventitial vessel layer that can be used towards inducing fibrosis, and the design-independent technical specifications for inducing fibrosis are in **Section 7.2**. The design-dependent technical specifications detail the parameters that were optimized for designing the adventitia.

3.1. Design-Independent Specifications

Table 3: Design-Independent Technical Specifications & Measurement Methods.

Specification Number	Specification	Measurement Method
A1	Greater than 80% of all cell types must remain alive for proper TEBV fabrication.	MTS assay analysis
A2	The distance between the individual adventitial layers and between the adventitial layer and the medial layer should be no more than 10 microns larger than the distance between each medial layer.	Analysis of multi-photon images to assess collagen alignment & cellular alignment of cell types using cell trackers
A3	RNA expression of smooth muscle cells shows a more contractile phenotype than those in a two-layered TEBV (> 1.5-	RT-PCR analysis

	fold RNA expression increase of α -SMA, calponin, and MYH11)	
--	---	--

Justifications:

A1: Cell viability

Seeding cells onto collagen and rolling them into TEBVs exposes them to significant mechanical stress. It was important to ensure that these cells remain alive after having been put in stressful environments to achieve the following design-independent technical specifications.

A2: Layer Incorporation into Existing TEBV Fabrication Method

To effectively model fibrosis, the fibroblasts in the adventitial layer had to be incorporated into the larger TEBV system to affect the medial and intimal layers. To measure this, the distance between adventitial layers, along with the distance between the adventitial and medial layers, was assessed. This distance should not be more than the distance between the individual medial layers; in other words, each individual layer in the media and adventitia should be evenly spaced, with no unusually larger gaps in between. In addition, the adventitial layer should not delaminate from the medial layer, but rather remain intact and bound around the medial layer.

A3: SMC Conversion from Synthetic to Contractile Phenotype

The biomechanical and biochemical changes resulting from adding an adventitial layer with a new cell type should push the SMCs within the TEBV towards a more mature, contractile phenotype [22]. This change was quantified by looking at RNA expression using Real-Time Polymerase Chain Reaction (RT-PCR).

3.2. Design-Dependent Specifications

Table 4: Design-Dependent Technical Specifications & Measurement Methods.

Specification Number	Specification	Measurement Method
B1	Fibroblast initial cell concentration is 250K-600K per 1.5 mm-diameter artery	MTS assay, live/dead cell imaging, and cell count post-digestion
B2	Collagen concentration of adventitial layer must be between 0.5-2 mg/mL	Dilute 5 mg/mL stock solution down to desired concentration within the specified range
B3	Adventitial layer thickness must be between 30-40% of the arterial wall thickness	Analysis of multi-photon images using second-harmonic imaging of collagen
B4	Adventitia can be made in batches of 9, and each adventitia in the batch satisfies technical specifications B1-B3	Differences between adventitia samples were assessed by analyzing the results from the measurements taken for B1-B3

Justifications

B1: Cell Density, Viability, & Proliferation

The adventitia cell concentration should mimic the cell concentration of native arteries and the concentration used to seed small-diameter TEBVs in existing literature. At a fibroblast (FB) seeding concentration between 250 K-600 K, the initial cell concentration must be optimized to provide enough cells to promote further proliferation, but not high enough to have an unnecessarily large cell density.

B2: Collagen Concentration

Adventitial tissue around native vessels is within 0.5-2 mg/mL [11]. In addition, previous TEBVs including FBs have been fabricated at collagen concentrations along this range [8][23]. If the concentration were lower, the layer would be too weak to provide support to the larger

vessel; if the concentration were higher; it would over-stiffen the vessel and make it challenging for cells embedded within the layer to migrate and proliferate. This concentration would provide structural support and a suitable environment to promote cell migration within the layer.

B3: Adventitial Layer Thickness

The adventitial layer in muscular arteries typically makes up 30-40% of the vessel wall thickness [24]. Similarly to collagen concentration, thinner adventitia are unable to provide necessary structural support, and thicker adventitia provide too much (as seen in fibrotic vessels).

B4: Reproducibility of Adventitia Engineering.

The fabricated adventitia should all satisfy technical specifications B1-B3 with limited variability between samples.

3.3. Design Approach

The design of the adventitial layer was engineered to mimic physiological parameters, and it was used to induce fibrosis within a tissue-engineered blood vessel (TEBV). The design process was focused on achieving the design-independent and design-dependent technical specifications.

Adventitia Fabrication

To design the adventitial vessel layer, two methods of fabrication could have been explored:

1. Molding method. As previously mentioned, the molding method developed in the Truskey lab gels a collagen solution containing smooth muscle cells (SMCs) and fibroblasts (FBs) into a conduit. This method can be adapted to fabricate the adventitia around a TEBV.

2. Rolling method. As previously mentioned, the rolling method used in the Chaikof lab involves seeding cells on a flat collagen sheet, then subsequently rolling the cell-laden sheet into a blood vessel conduit. For adventitia fabrication, a similar technique was employed. After rolling the tunica intima and tunica media into a TEBV, the FBs were seeded on a shortened layer of collagen (**Figure 8**). This sheet was subsequently rolled around the fabricated TEBV, with its shortened length allowing for a thin adventitial layer with precise circumferential collagen alignment.

Table 5: Design-Dependent Technical Specification Pugh Matrix for Design Approaches.

Design-Dependent Technical Specifications	Importance Weighting	Molding Method	Rolling Method
Cell Viability	5	-1	+1
Adventitial Layer Thickness	4	0	+1
Collagen Concentration	3	+1	-1
Totals		-2	6

Cell Density, Viability, & Proliferation. Previous work in the Chaikof Lab has statistically determined that cell viability is significantly lower in molded TEBVs than in rolled TEBVs, as detailed in the **Previous Work** section. When choosing which design approach to pursue, it was important to assess whether this previously determined conclusion holds true in the context of engineering the adventitial layer.

Collagen Concentration. Due to the nature of the molded adventitia fabrication process, it is easy to adjust the collagen concentration of the layer. A collagen solution can be diluted to a precise concentration before being poured into a syringe for gelation. Conversely, it is more

difficult to control the collagen concentration of the adventitial layer using the rolling method. The extrusion process currently used to create collagen sheets that are later rolled into TEBVs produces ultrathin sheets at a 5 mg/mL collagen concentration; if the concentration were to be lowered, it may be difficult to obtain an ultrathin, uniform collagen sheet.

Adventitial Layer Thickness. Previous work in the Chaikof Lab has determined that molded TEBVs have lumpy, nonuniform vessel walls with randomly aligned collagen fibers. This could make it difficult to achieve a homogenously thick adventitial layer that makes up 30-40% of the total vessel wall thickness. Conversely, the rolling method results in a smooth, circumferentially aligned TEBV. This method was chosen because it is more likely to achieve a uniformly thick adventitial layer.

Layer Incorporation into Existing TEBV Fabrication Method. The Chaikof Lab has employed the rolling method to construct the inner two layers of blood vessels. Because of how these TEBVs are rolled, air pockets can get in between rolled layers, and the outer layers can become delaminated if not rolled carefully. These TEBVs are made up of closely wound layers that mimic the aligned collagen fibers seen in native tissues. Native vessels also contain an adventitial layer directly against the medial layer, with its own layers and cell types within. It was important to fabricate a TEBV that has 3 distinct layers that are flush against each other, as well as to avoid any delamination of the outer layers after the rolling process.

Reproducible Adventitia Fabrication. The molding method is a rapid, high-throughput process that is able to produce a large volume of adventitia layers with a large amount of variability. The rolling method is a much slower process that would produce a smaller volume of adventitia that better adhere to the design-dependent technical specifications.

From the results of the Pugh matrix, it was anticipated that the rolling method would more successfully meet the technical specifications for engineering the adventitia. Thus, the rolling method was used to engineer the adventitial layer.

Adventitia Design Parameter Optimization

To systematically optimize Design-Dependent Technical Specifications B1-B3, collagen concentration, fibroblast cell density, and adventitial sheet length were varied and optimized. Sample groups were fabricated in groups of 3 and were characterized according to their adherence to the design-independent technical specifications.

Collagen Concentration. The adventitia was fabricated to adhere to Design-Dependent Technical Specification B2 and maintain the collagen concentration of the adventitial layer to be between 0.5-2 mg/mL. However, achieving this concentration proved difficult for two reasons: first, it was difficult to extrude ultrathin collagen sheets within this collagen concentration. There were observable tears in the extruded sheets, and the collagen sheet was not uniformly thick. Secondly, the intact collagen sheets that could be extruded at the higher end of this concentration range could not be rolled into the adventitial layer because the sheet would consistently tear during the rolling process. Thus, a 5 mg/mL collagen concentration was used to engineer the adventitial layer to avoid these problems. In addition, the ultrathin collagen sheet extrusion has been extensively characterized at this concentration in the Chaikof Lab [9]. See more of the explanation in **Section 6.1**.

Fibroblast Cell Density. The density of FB cells in the adventitial layer was optimized both to adhere to Design-Dependent Technical Specification B1 (that there must be 250K-600K FBs per adventitial layer) and to minimize the number of FBs per adventitia. Due to the slow cell

growth of the human aortic adventitial FBs cultured in the Chaikof Lab, it was important to look at minimizing this number to maximize replicate output.

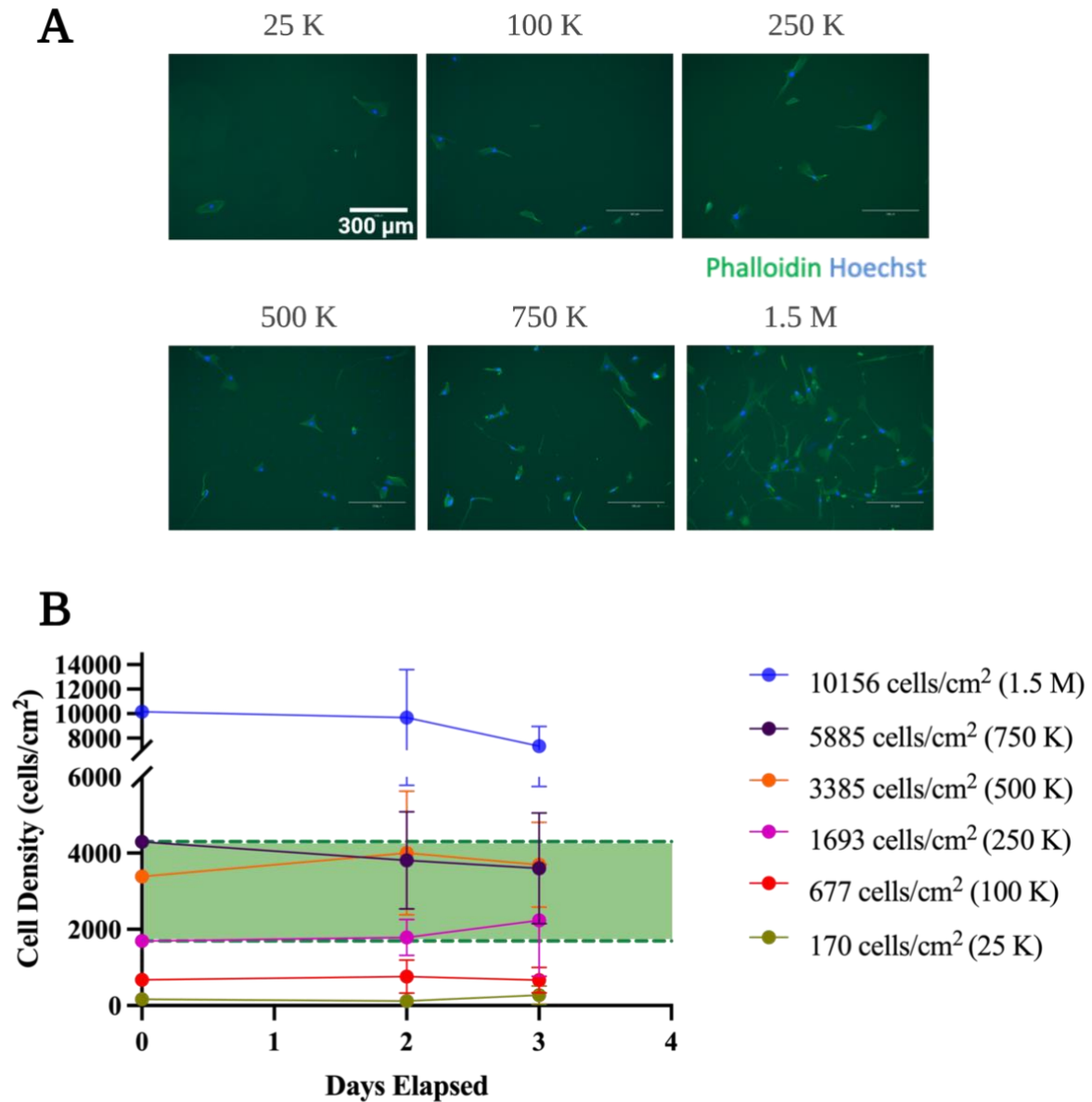


Figure 4: Evaluation of fibroblast growth over 3 days at increasing cell densities. (A) Confocal images of Phalloidin and Hoechst-stained fibroblasts at increasing cell densities. The densities above the image represent the total number of cells that would be added to one adventitia based on the layer area. **(B)** The change in cell density over 3 days. Measurements were taken on Days 0, 2, and 3. Legend corresponds to

the cells per cm² in the 6-well plate, with the cells per adventitia in parentheses. The green shaded region represents the acceptable range denoted in Technical Specification B1.

Adventitial Sheet Thickness. Adventitial sheet thickness was designed to adhere to Design-Dependent Technical Specification B3 so that it comprised 30-40% of the total arterial wall thickness. To achieve this range, a mathematical model was employed to calculate the sheet length necessary to adhere to this specification.

Mathematical Modelling of Adventitial Sheet Length

To calculate the appropriate adventitial sheet length range, the following equation was used:

Equation 1: Adventitial sheet length calculation with E* error factor.

$$Sheet\ Length = \frac{(R_o^2 - R_I^2)}{T} * \pi E^*$$

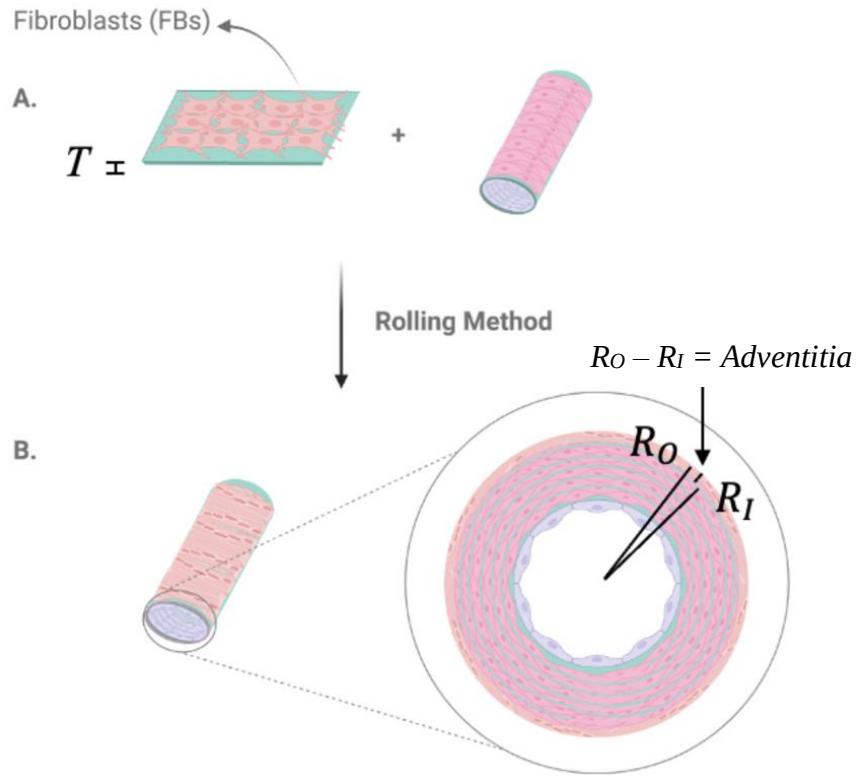


Figure 5: The R_I and R_O of a tri-layered TEBV. (A) A fibroblast-laden sheet with a thickness $T = 4.8 \mu\text{m}$ was rolled in around a two-layered TEBV to create the adventitial layer. (B) The difference between the outer radius (R_O) and the inner radius (R_I) equals the thickness of the adventitial layer. The adventitia sheet length was calculated using the above equation, then fabricated and rolled around a two-layered TEBV.

where R_O is the total radial length of the tri-layered TEBV, R_I is the radial length of the TEBV up to the adventitial layer, and T is the thickness of the collagen sheets. Due to a discrepancy between the mathematically calculated thickness and the experimentally measured thickness, an E^* error factor was incorporated into the equation to account for this difference.

The E^* factor was calculated to be 2.09; in other words, the experimentally measured thickness of the TEBV was about two times the mathematically calculated thickness. This difference could be attributed to several sources, such as (but not limited to) air pockets formed during the rolling process, cell layer thickness that is not considered in this calculation, and other fabrication variability. It is known that T is $4.08 \mu\text{m}$, R_I is $430 \mu\text{m}$, and R_O should be $430 +$

430*(30%-40%) μm to satisfy the technical specifications. With this, the range of sheet lengths tested to satisfy the technical specifications was 5-11 cm. To cover this range, 3 lengths at 5 cm, 7.6 cm, and 11 cm were chosen to fabricate adventitia that comprised 28%, 34%, and 44% of the total arterial wall thickness respectively.

Cell Viability MTS Standard Curve Design

Design-Independent Technical Specification A1 states that fibroblasts (FBs) in the adventitial layer must remain greater than 80% viable after the rolling process. To effectively measure whether the FBs in the adventitial layer were viable after rolling, an (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay was employed. However, the MTS reagent could only be added to the entire tissue-engineered blood vessel (TEBV) construct, measuring the viability of both the SMC medial layer and the FB adventitial layer. To account for this, an MTS assay standard curve was constructed using a co-culture of FBs and SMCs to mimic the co-culture in the TEBVs containing a medial and adventitial layer. The standard curve was built using a FB cell density range spanning below and above the number of FBs per adventitia as per Technical Specification B1 (**Table 6**, column 1). This FB seeding density was translated from the number of FBs seeded per the area of an adventitial sheet, to the number of FBs seeded in the much smaller area of a well on a 96-well plate (**Table 6**, column 2). SMCs were seeded at a FB:SMC ratio of 1:3 (**Table 6**, column 3), similar to what would be observed in the tissue-engineered blood vessels (TEBVs) containing an adventitial layer making up 34% of the total arterial wall thickness. The values of the numbers of cells seeded per well can be found in **Table 6**. The standard curve experimental setup is further explained in **Section 4.3.1**.

Table 6: Number of fibroblasts (FBs) and smooth muscle cells (SMCs) seeded per well. Well positions (Column 1) correspond to the 96-well plate shown in **Figure 6**. From the number of fibroblasts seeded in one adventitia (Column 2), the corresponding cell density of a 1:3 FB:SMC ratio in a 96-well plate was calculated (Columns 3-4).

Well Positions	Fibroblast Cell Number Per Adventitia	Number of FBs Seeded	Number of SMCs Seeded
D1-F1	25 K	0.233 K	0.569 K
D2-F2	100 K	0.93 K	2.268 K
A3-C3	250 K	2.325 K	5.67 K
A4-C4	500 K	4.65 K	11.341 K
A5-C5	750 K	6.975 K	17.012 K
A6-C6	1 M	9.3 K	22.683 K
A7-C7	1.5 M	13.95 K	34.024 K

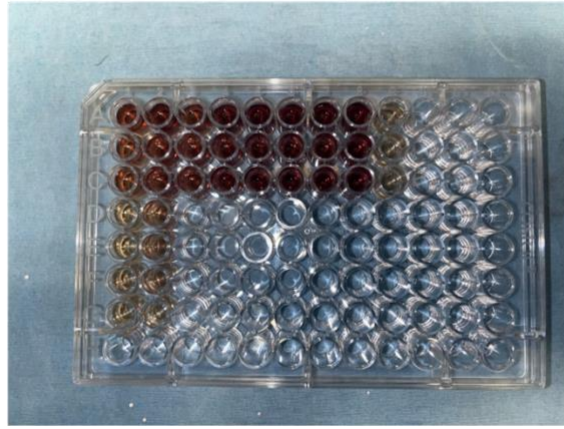
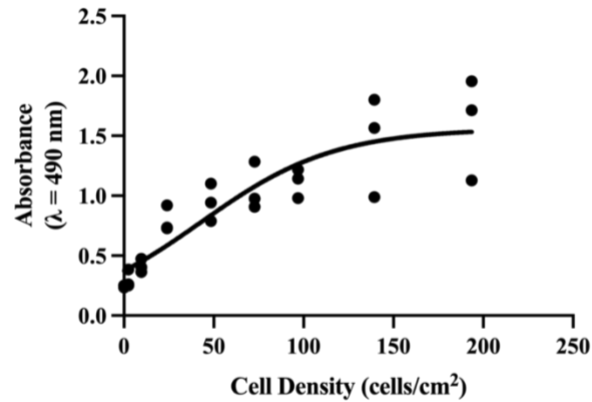
A**B**

Figure 6: MTS Assay Standard Curve. (A) 96-well plate containing the number of FBs and SMCs detailed in **Table 6** columns 3 & 4. The MTS reagent was immediately added to each well, and the plate was imaged after 4 hours. (B) The MTS standard curve plotting the measured absorbance after 4 hours of each well containing the MTS reagent against the seeded co-culture cell density. It was assumed that all cells in each well were viable immediately after seeding. A logarithmic growth curve was fitted to the data to generate the standard curve, $n = 3$ replicates per cell density.

After generating the standard curve, cell viability of experimental samples could be measured. The MTS reagent was added to the media around the TEBV construct, and the solution's absorbance was measured after 4 hours. This absorbance could be translated to a measured cell density using the standard curve in **Figure 6**, and the percent cell viability was calculated using the following equation:

Equation 2: Percent cell viability calculation using known seeding density and measured cell density from the generated standard curve.

$$\% \text{ Cell Viability} = \frac{\text{Measured Cell Density}}{\text{TEBV Cell Seeding Density}} * 100$$

The percent cell viability was measured against Design-Independent Technical Specification A1 to ensure that greater than 80% of the fibroblasts remain viable after the rolling process.

3.4. System Diagram

The process of developing a tri-layered tissue-engineered blood vessel requires 2 phases: tissue-engineered blood vessel (TEBV) fabrication and adventitial engineering. Looking forward, the tri-layered TEBV will be used for modeling fibrosis in a 3D blood vessel environment.

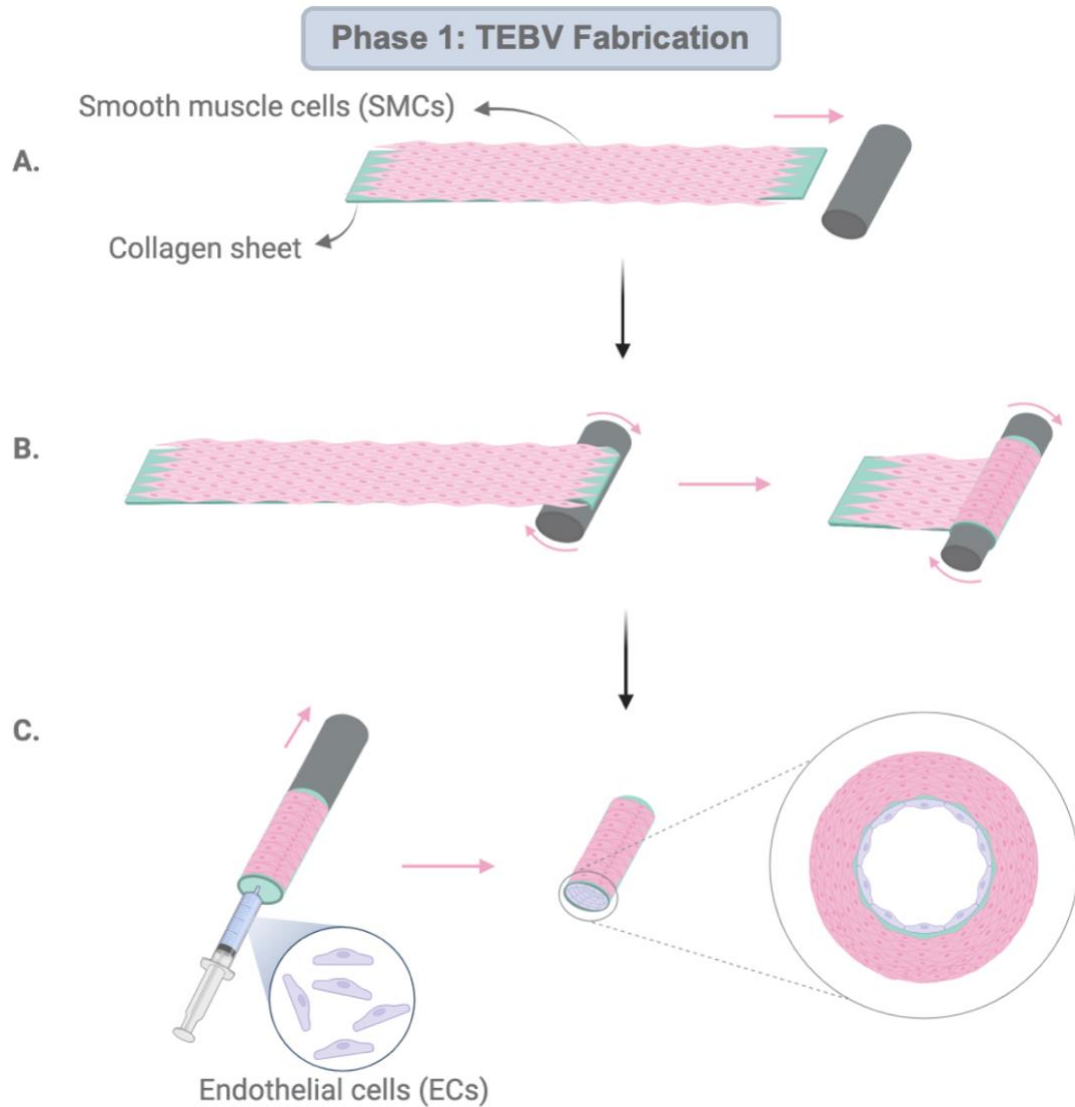


Figure 7: Schematic of TEBV fabrication, phase 1 of the design process. (A) The first step of the design process involves fabricating TEBVs for further use. First, SMCs are embedded atop an ultrathin collagen sheet [9]. (B) The cell-laden sheet is then rolled around a mandrel into a vessel conduit to fabricate the tunica media. (C) The mandrel is removed, leaving the vessel conduit behind. A solution containing ECs is injected into the lumen of the vessel, forming a monolayer of ECs (the tunica intima).

The Chaikof Lab has an established protocol for fabricating the arterial tunica media (shown in pink in **Figure 7**). Smooth muscle cells (SMCs) are embedded on top of a collagen sheet, and the cell-laden collagen sheet is rolled around a mandrel to fabricate the tunica media. These constructs are further endothelialized with human aortic endothelial cells (ECs), and this protocol was being optimized concurrently alongside this project.

Phase 2: Adventitial Engineering

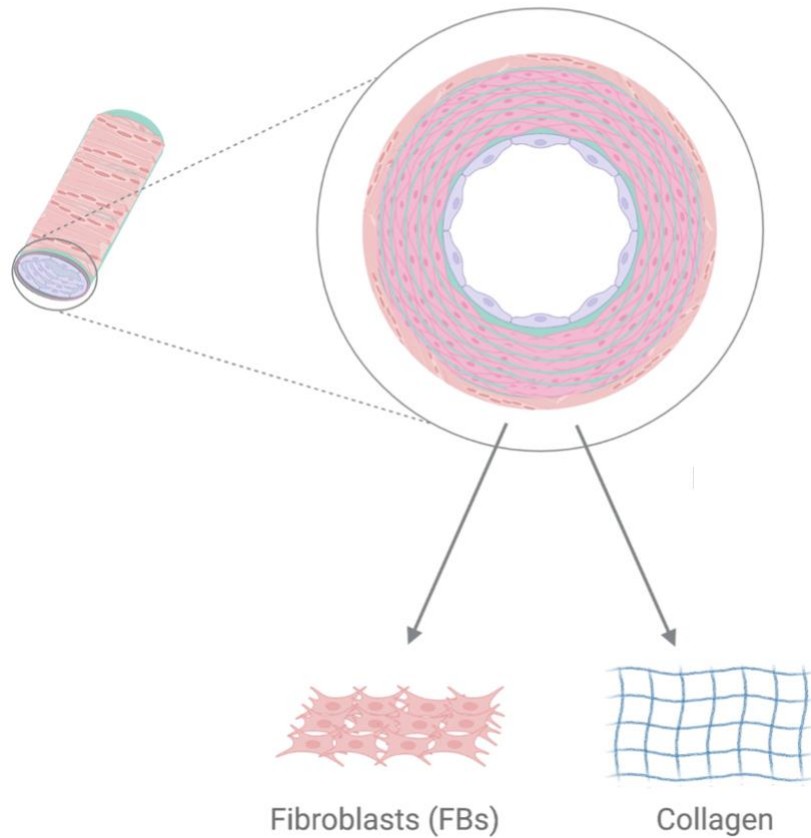


Figure 8: Adventitial Engineering, phase 2 of the design process. (A) The adventitial layer was added around the fabricated TEBV from phase 1. The rolling method was used to fabricate the adventitial layer. After building the adventitial layer, it was characterized by its adherence to the design-independent technical specifications.

This project focused on phase 2, adventitial engineering. The adventitial layer was constructed using collagen and human aortic adventitial fibroblasts (FBs), and it was added to the TEBV fabricated in phase 1. The adventitial layer was added onto the two-layered TEBV using the rolling method as the most promising fabrication method, as explained in **Section 3.3**.

Phase 3: Modeling Fibrosis

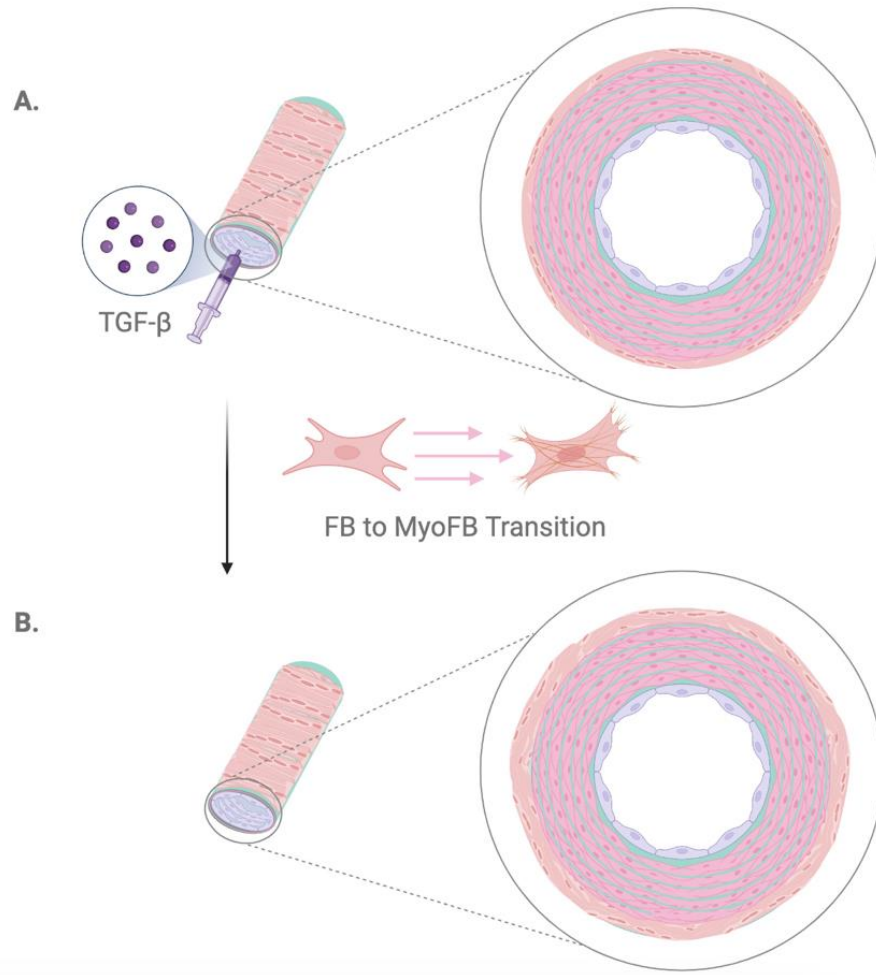


Figure 9: Modeling fibrosis, phase 3 of the design process. This phase synthesizes the work done in phase 1 & phase 2. **(A)** TGF- β , a pro-fibrotic agent, will be perfused through the TEHV lumen. This will stimulate FBs to transition to MyoFBs, resulting in a fibrotic blood vessel microenvironment. This change should adhere to the design-independent technical specifications detailed in **Section 7.2**. **(B)** The work of achieving phase 3 will continue in the lab. A fibrotic TEHV will be created after exposure to a pro-fibrotic agent. Each design-independent technical specification listed will be tested to see if the quantified specification is met. If not, phase 2 will be iterated, then phase 3 will be re-tested.

The goal of engineering a tri-layered TEHV is to create a fibrotic blood vessel microenvironment. Though out of the scope of this project, preliminary work was performed towards this end (**Section 6.4.2**). This work will be continued in the lab towards effectively modeling fibrosis.

4. Materials & Methods for Fabrication

This tissue-engineered blood vessel (TEBV) model was built at the Chaikof Lab in the Beth Israel Deaconess Medical Center (BIDMC) Longwood Medical Area. The rolling TEBV fabrication method detailed in **Section 3.4** was used to create the innermost layers of the TEBV. As per the results in the Pugh Matrix (**Table 5**), the rolling method was also used to engineer the adventitial layer.

4.1. Materials Preparation

4.1.1. Cell Culture

Primary human aortic smooth muscle cells (Lonza) were cultured in Smooth Muscle Cell Growth Media (Lonza) supplemented with 20% FBS (Lonza), non-essential amino acids (Lonza), 1% Pen/Strep (Thermo Fisher), and 0.1% Fungin (Thermo Fisher). Cells in passages 2-10 were used for all experiments.

Primary human aortic adventitial fibroblasts (Lonza) were cultured in Stromal Cell Growth Medium (Lonza), supplemented with 5% FBS (Lonza), 1% Pen/Strep (Thermo Fisher), and 1% Fungin (Thermo Fisher). Cells in passages 2-10 were used for all experiments.

4.1.2. Collagen Solution & Buffer Preparation

A collagen solution (5 mg/mL) was prepared by dissolving lyophilized Type I monomeric collagen (Rockland) in deionized water (pH 2) containing green food dye (Club House, Canada) for visualization. The solution was stirred continuously at 4°C for 24 hours to obtain an acidic collagen solution. The rapid gelation of collagen solution was induced by the addition of the buffering salts and polyethylene glycol (PEG), a molecular crowding and gelation triggering agent, to deionized water. Following formation, collagen sheets were incubated in

phosphate buffer to induce fibrillogenesis. The Fiber Incubation Buffer (FIB) was prepared in deionized water and consisted of sodium chloride (7.89 mg/mL), dibasic sodium phosphate (4.26 mg/mL), and Tris (10 mM), and the pH was adjusted to 7.4 [25].

4.1.3. Medial & Intimal TEBV Fabrication

Collagen sheets were fabricated using an established protocol in the Chaikof lab [9]. After incubation in the Fiber Incubation Buffer (FIB), the collagen sheets were sterilized overnight. 7 mL of a solution containing 1.38×10^6 SMCs were seeded on top of each collagen sheet and cultured at 37°C for 72 hours. The cell-laden collagen sheets were rolled around a split mandrel into a TEBV using a custom rotor and conveyor belt system. Rolled TEBVs were cultured in 6-well plates containing 5 mL of SMC media per well for 7 days.

The statically cultured TEBVs were perfused with a 1 mL solution containing 800K primary human aortic endothelial cells. The TEBV was rotated 180 degrees over 24 hours to evenly coat the inner lumen with endothelial cells.

4.2. Adventitia Engineering

Adventitia Crosslinking & Fabrication

Collagen sheets were fabricated using an established protocol in the Chaikof lab [9]. After incubation in the Fiber Incubation Buffer (FIB), the collagen sheets are crosslinked for 1 hour at 37°C using a 6 mg/mL genipin solution (Sigma Aldrich). The collagen sheets are then cut to the desired length and sterilized overnight.

4.3. Methods of Adventitia Characterization

4.3.1. Cell Viability & Proliferation

To quantify cell viability, an (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay was performed as similarly described in ASTM F3504-21 (**Appendix 7.3.1**).

MTS Standard Curve. First, a standard curve was generated using a 2D fibroblast (FB)-smooth muscle cell (SMC) co-culture in 96-well plates. The standard curve was built using the method described in **Section 3.3**. 20 μL of the MTS assay reagent (Abcam) was added to each well containing 200 μL of media. After 4 hours, a plate reader was used to measure the absorbance of the solution in each well at $\lambda = 490 \text{ nm}$, and this absorbance was plotted against the known cell seeding density within the 96-well plate. **Figure 6** includes the plate setup of the MTS assay, as well as the generated standard curve.

A similar experiment was conducted for TEBV samples. The absorbances measured for the TEBV samples were translated to a known cell density using the previously generated standard curve, and cell viability was measured by comparing this measured cell density to the known cell density seeded into the TEBVs as shown in **Equation 2**.

4.3.2. Layer Radial Thickness

The radial cross-sectional thickness was measured using multi-photon second harmonic and confocal microscopy (Leica SP5 X) to image the collagen and cell layers. TEBV constructs were placed on their side so that their cross section was perpendicular to the microscope. ImageJ analysis was used to measure the average radial thickness of each layer. See **Section 6.3** for further discussion on the imaging method used for assessing cross-sectional layer thickness.

4.3.3. RT-PCR gene expression analysis

CellTracker™ Fluorescent Dye

CellTracker™ Red Dye (Thermo Fisher) and CellTracker™ Deep Red Dye (Thermo Fisher) were added to each cell population for imaging and fluorescence-activated cell sorting. The cells were pelleted in a 15 mL falcon tube. Media was aspirated from the pellet, and the CellTracker™ Red Dye (emission = 577/602 nm) and CellTracker™ Deep Red Dye (emission = 630/650 nm) were added in a 1:1000 dilution to the FBs and SMCs respectively. Cells were incubated at 37°C for 30 minutes, then CellTracker™ staining solution was removed from the cell pellets. The cells were washed with Phosphate Buffer Solution (PBS) three times, and the cells were seeded onto collagen sheets as described in **Section 4.1.3**.

TEBV Construct Digestion

TEBV construct digestion was used to retrieve FBs and SMCs embedded within the constructs for further qPCR analysis. The construct enzymatic digestion was adapted from Truskey et al. [23] and Deng et al. [26]. A solution of 1 mg/mL collagenase IV (Thermo Fisher) and 1% DNase I (Sigma Aldrich) was prepared. Constructs were submerged in 1 mL of this solution and shaken at 37°C for 30 minutes. The cells were pelleted and resuspended in 0.3 mL of PBS.

Fluorescence-Activated Cell Sorting (FACS)

Fluorescent-Activated Cell Sorting (FACS) was used to segregate cell populations from digested constructs (CytoFLEX SRT). A polygonal forward scatter threshold was drawn to eliminate signals arising from debris while allowing for retrieval of FB and SMC populations. PE (Phycoerythrin, emission peak = 574 nm) and APC (Allophycocyanin, emission peak = 660

nm) were used as the fluorescent channels to sort the cell populations, since their emission spectra are similar to CellTracker™ Red Dye and Deep Red Dye respectively. FBs loaded with CellTracker™ Red Dye were gated as PE^{pos}APC^{neg}, while SMCs loaded with CellTracker™ Deep Red Dye were gated as PE^{neg}APC^{pos}. 10,000 events were recorded for each sample sort. All relevant Side Scatter (SSC) vs Forward Scatter (FSC) and PE vs APC fluorescence density plots can be found in **Section 5.3** and **Section 7**.

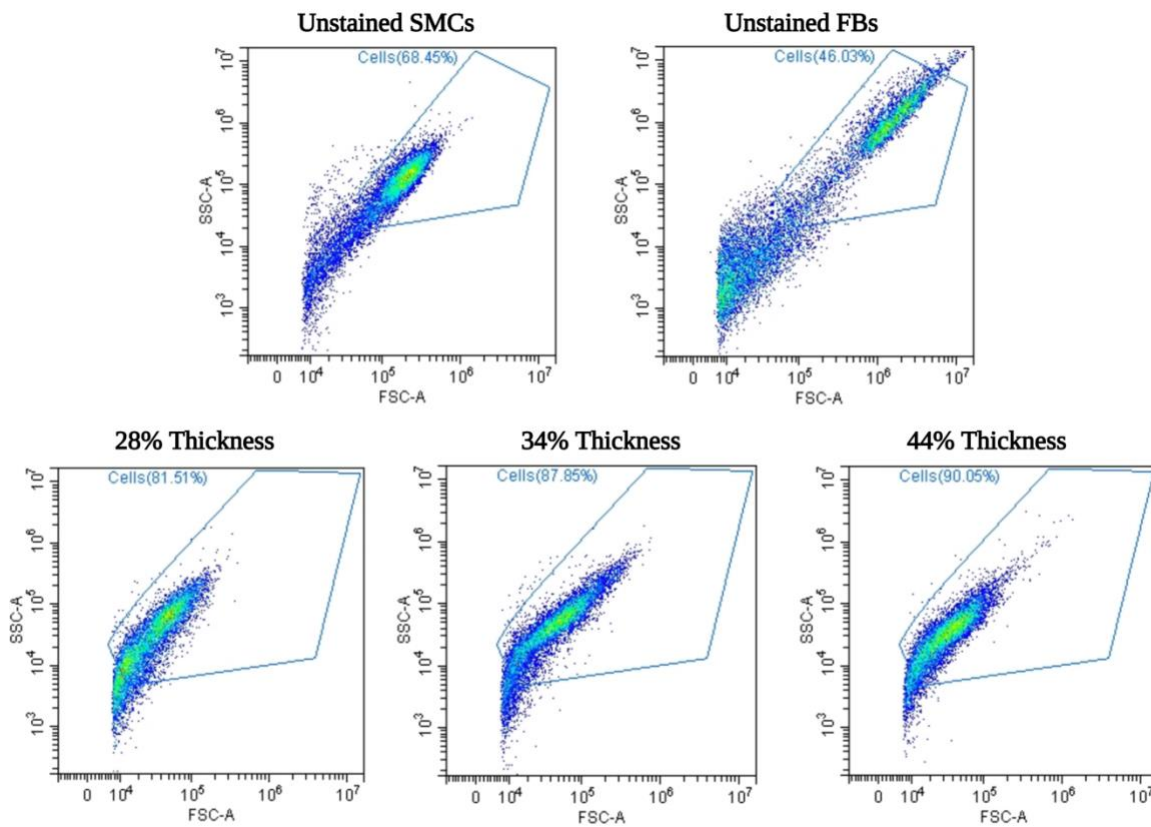


Figure 10: SSC vs. FSC density plots from FACS analysis. Each dot or point on the plot represents an individual particle that passed through the laser. A forward scatter threshold was applied to remove debris from the sorted samples. Plot titles in the second row correspond the expected percent adventitial thickness of the total arterial wall thickness.

RNA Isolation, cDNA Synthesis, and Reverse Transcriptase-PCR Analysis

RNA isolation was performed using a Qiagen RNeasy Mini Kit (Qiagen) following the RNeasy Mini protocol [27]. The concentration and purity of RNA samples were measured using the NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer (Thermo Scientific). cDNA synthesis was subsequently performed using 16 µL of the isolated mRNA samples and nuclease-free water, and 4 µL of the H Maxima cDNA Synthesis Master Mix (Thermo Fisher) as per the protocol [28]. Each cDNA sample was diluted to 0.2 ng/µL. TaqMan probes *α-SMA*, *MYH11*, *COL1A1*, and reference gene *RPLP0* and TaqMan Master Mix were purchased from Thermo Fisher. Reactions were performed in technical triplicates using a 384-well plate. As per the protocol [29], each 20 µL reaction contained 10 µL of TaqMan Master Mix, 1 µL of the TaqMan probe, and 9 µL of sample cDNA and nuclease-free water. The ViiA 7 Real-Time PCR system (Applied Biosystems) was used to perform RT-PCR using the hold and cycle stages specified for TaqMan reagents in the protocol. RT-PCR amplification curves can be found in **Appendix 7.1**.

5. Evaluation of Results

To characterize the fabrication and incorporation of the adventitial layer into the tissue-engineered blood vessel (TEBV) model, (1) cell viability, (2) layer thickness, and (3) the impact of the adventitial layer on the medial layer, measured by the change in expression of key smooth muscle cell genes in TEBV samples containing an adventitial layer, were assessed. Each of these measured results was compared to the expected values detailed in Design-Independent Technical Specifications A1-A3 to assess whether the specification was successfully met.

5.1. Cell Viability Analysis

Cell viability of FBs and SMCs after TEBV fabrication culture was assessed. TEBVs were statically cultured for 7 days, and the MTS reagent was added to the sample media in a 1:10 dilution. After 4 hours of incubation with the MTS reagent, replicates of the sample media solution were taken from each well, transferred to a 96-well plate, and the absorbance of a sample was measured at $\lambda = 490$ nm using a plate reader. The measured absorbance was translated to a cell density using the constructed MTS standard curve described in **Section 3.3**, and percent cell viability was calculated by using **Equation 2**.

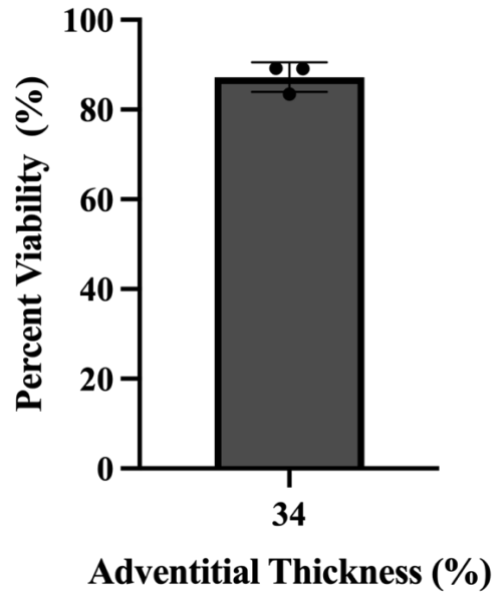


Figure 11: Cell viability of TEBVs containing the medial and adventitial layers. The samples contain an adventitia that makes up 34% of the total arterial wall thickness. Data are mean $\pm$ standard deviation from the mean, n = 3 TEBV constructs.

Mean cell viability was found to be 87.23%, with a standard deviation of 3.30%. This adheres to the Technical Specification A1, requiring for 80% the number cells originally seeded in the TEBV to remain viable through the culturing process. For the sample group with an adventitial layer that constituted 34% of the total arterial wall thickness, all samples contained a co-culture of fibroblasts and smooth muscle cells that were greater than 80% viable. It was assumed that TEBV constructs containing adventitial layers that made up 28% and 44% of the total arterial wall thickness would be similarly viable, since they are similarly fabricated and cultured.

5.2. Layer Thickness

To measure the layer thickness, smooth muscle cell and fibroblast populations were incubated with CellTracker™ Deep Red dye and Red dye for 1 hour, respectively. TEBVs were subsequently fabricated using these cells and statically cultured for 7 days. Adventitial layer

thickness was assessed by imaging the cross-section of the TEBV constructs, then measuring the lengths of the medial and adventitial layers using ImageJ.

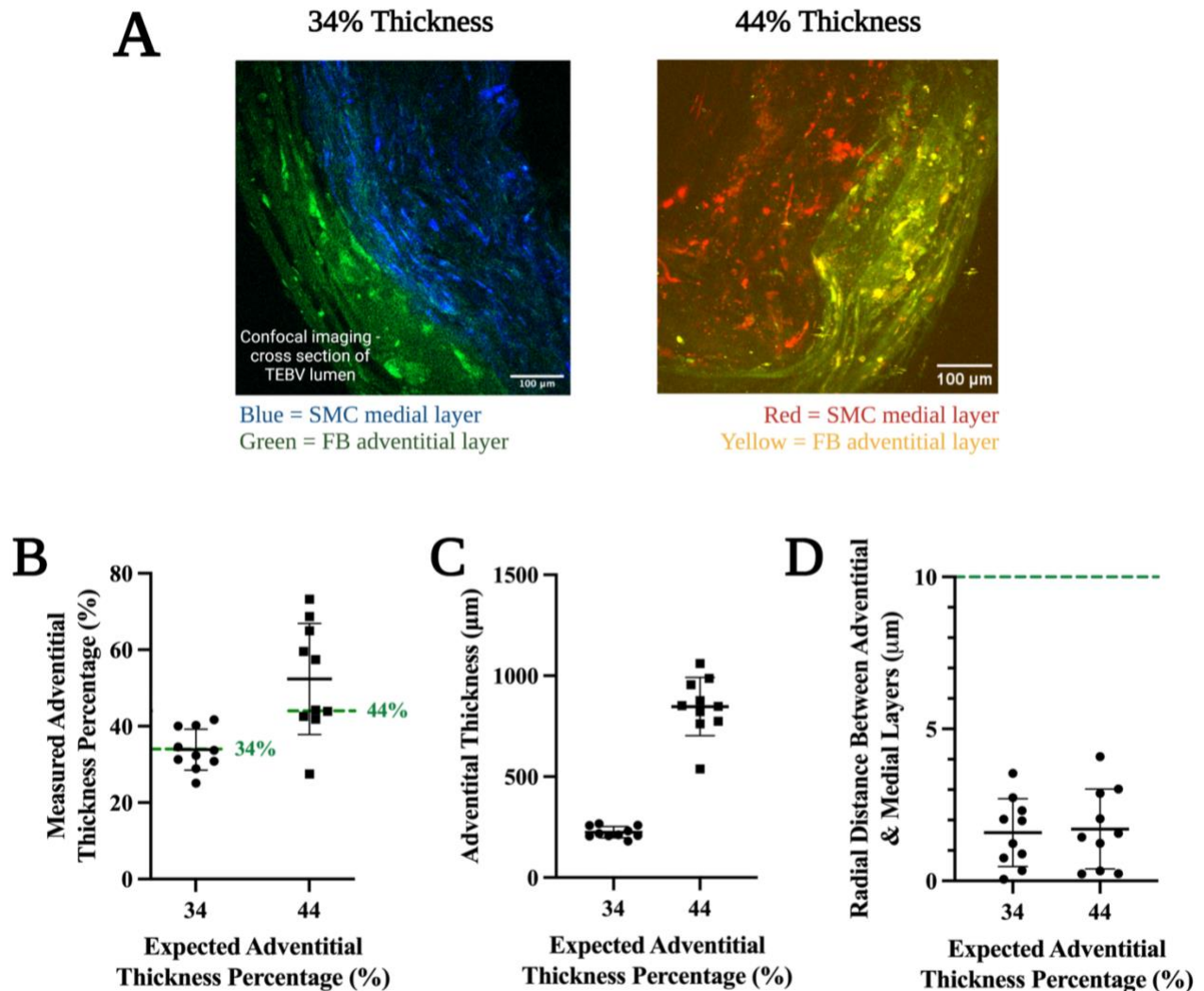


Figure 12: Evaluation of Adventitial Layer Thickness in TEBV constructs. (A) Image slices taken from a 3D image stack of TEBV constructs containing an expected adventitial thickness of 34% (left) and 44% (right) of the total arterial wall thickness. (B) Measured adventitial thickness percentage measured in two sample groups. Dotted green lines represent expected adventitial thickness percentage. (C) Measured adventitial thickness lengths (μm) measured in two sample groups. (D) Measured radial distance between the adventitial and medial TEBV layers. Data are mean $\pm$ standard deviation from the mean, $n = 3$ images per sample group ($n = 10$ total).

For the sample group containing an expected adventitial thickness of 34% of the total, the mean measured percent adventitial thickness was 33.87%, with a standard deviation of 5.37%. These results adhere to Technical Specification B3, stating that the adventitial thickness should comprise 30-40% of the total arterial wall thickness.

For the sample group containing an expected adventitial thickness of 44% of the total, the mean measured percent adventitial thickness was 52.39%, with a standard deviation of 14.53%. The mean measured thickness is above the range stated in Technical Specification B3, and the standard deviation from the mean is larger than that of the other sample group. This discrepancy may be due to the imaging method used, as described in **Section 4.3.2**, and the pitfalls are described in **Project Limitations**.

For both sample groups, the mean radial distance was lower than 10 μm , with an average distance of 1.586 μm for the sample with a 34% expected thickness and 1.708 μm for the sample with a 44% expected thickness. These results adhere to Design-Independent Technical Specification A2.

5.3. Gene Expression Analysis

Gene expression analysis of smooth muscle cell populations cultured in a TEBV construct containing an adventitial layer were analyzed using Real-Time Polymerase Chain Reaction (RT-PCR). TEBV constructs were digested, smooth muscle cells were sorted from fibroblasts using FACS, and subsequently RNA was isolated from smooth muscle cells populations, and RT-PCR was analyzed using generated cDNA.

TEBV Digestion & Fluorescence-Activated Cell Sorting (FACS)

Prior to TEBV fabrication, smooth muscle cell and fibroblast populations were incubated with CellTracker™ Deep Red dye and Red dye for 1 hour, respectively. TEBVs were subsequently fabricated using these cells and statically cultured for 7 days, then digested for 30 minutes in a 1 mg/mL collagenase IV and 1% DNase I solution. The fibroblasts and smooth muscle cells in the resulting cell suspension were sorted using FACS (CytotFLEX SRT), with the PE channel used to sort fibroblasts loaded with CellTracker™ Red dye, and the APC channel used to sort smooth muscle cells loaded with CellTracker™ Deep Red dye. FBs loaded with CellTracker™ Red Dye were gated as PE^{pos}APC^{neg}, while SMCs loaded with CellTracker™ Deep Red Dye were gated as PE^{neg}APC^{pos}.

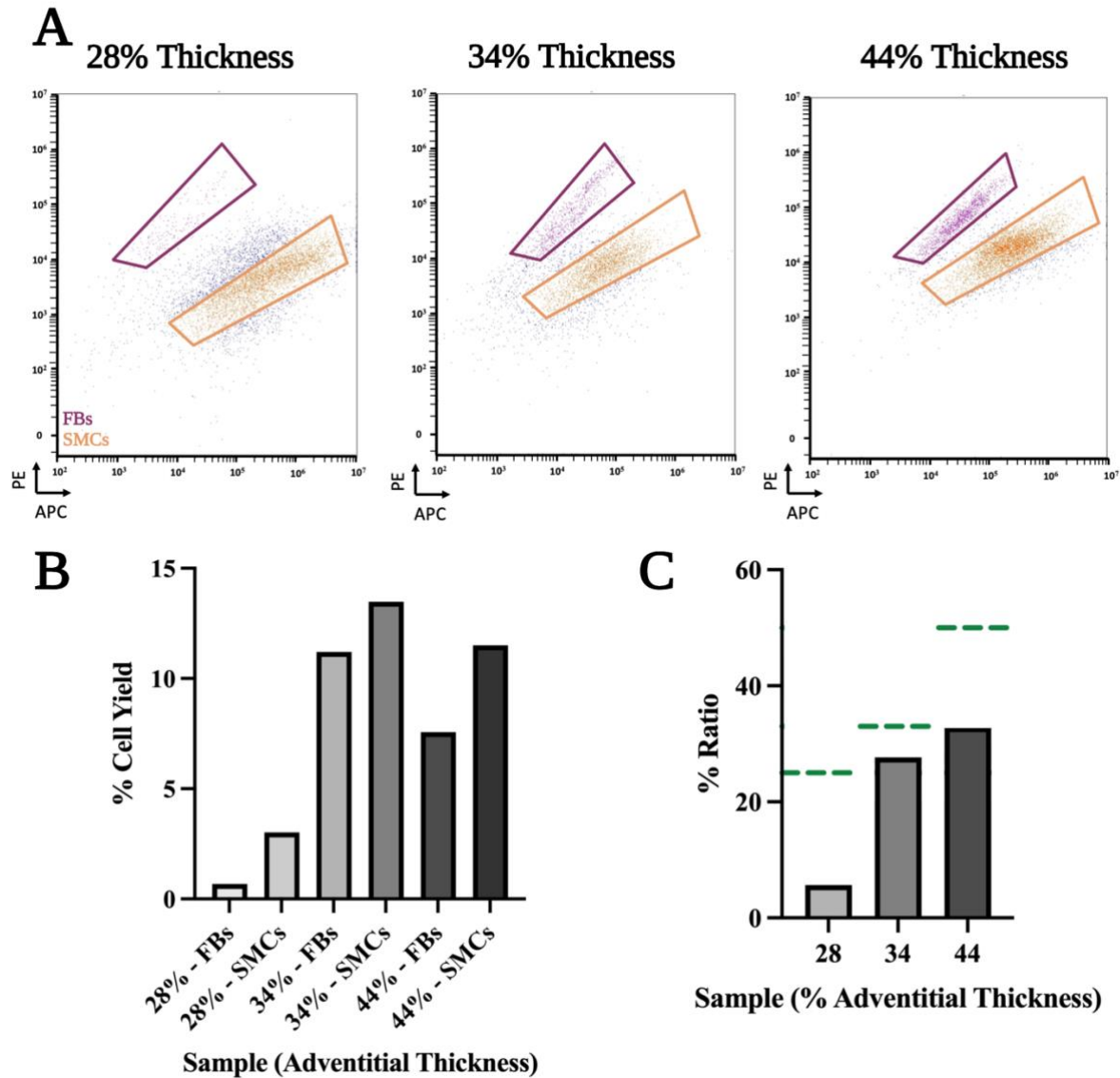


Figure 13: Evaluation of Fluorescence-Activated Cell Sorting (FACS) retrieval of fibroblast and smooth muscle cell populations. (A) Side Scatter (SSC) vs Forward Scatter (FSC) density plots depicting the distribution of fibroblasts and smooth muscle cells for sample groups containing an adventitial layer comprising 28%, 34% and 44% of the total arterial wall thickness. Fibroblasts are gated in purple, and smooth muscle cells are gated in orange. **(B)** The percent yield of fibroblasts (FBs) and smooth muscle cells (SMCs) retrieved from FACS for the 28%, 34% and 44% adventitial thickness sample groups. **(C)** The percent ratio of FBs:SMCs retrieved from FACS for the 28%, 34% and 44% adventitial thickness sample groups. The green lines above each bar denote the percent ratio of FBs:SMCs originally seeded in the TEBV construct, $n = 1$ TEBV per adventitial thickness.

For the 28% thickness sample, 3.49% of the single cells detected were grouped within the fibroblast gate, and 60.42% were grouped within the smooth muscle cell gate. For the 34%

thickness sample, 16.91% of the detected cells were fibroblasts, and 63.36% were smooth muscle cells; and for the 44% thickness sample, 23.55% of the detected cells were fibroblasts, and 63.83% were smooth muscle cells.

Cell yield for all sample groups was calculated by counting the number of fibroblasts or smooth muscle cells retrieved after FACS to the number of the respective cell types that were originally seeded into each TEBV construct (see **Table 8**). Percent ratio of fibroblasts (FBs) to smooth muscle cells (SMCs) was calculated using the cell counts retrieved from FACS. The dotted green lines above the bars represent the FB: SMC seeding ratio for each sample.

RNA Isolation, cDNA Synthesis, and RT-PCR

RNA isolation was performed (QIAGEN RNeasy Mini Kit) on the fibroblast and smooth muscle cell samples retrieved from FACS. cDNA synthesis was subsequently generated using the RNA samples (H Maxima cDNA Synthesis Kit).

RT-PCR (TaqMan) was performed using 1 ng of the generated cDNA from SMC samples. The reference gene used was *RPLP0* (ribosomal protein lateral stalk subunit P0), and the genes of interest were *COL1A1* (collagen type 1 alpha 1), *ACTA2* (aorta smooth muscle actin alpha 2), and *MYH11* (myosin heavy chain 11). Smooth muscle cells without CellTracker™ dye and monocultured in 2D flasks were used as a control. *ACTA2* and *MYH11* are smooth muscle cell genes known to increase in expression in smooth muscle cell populations exhibiting a more contractile, mature phenotype. *COL1A1* is known to increase in expression in smooth muscle cell populations with more synthetic, immature phenotypes. The hypothesis was that *ACTA2* and *MYH11* expression would increase, and *COL1A1* expression would decrease, in smooth muscle cell populations cultured in 3D TEBV environments containing an adventitial layer because the fibroblast layer would push them towards a more contractile, mature phenotype due to

biomechanical forces due to compressive forces from the adventitial layer onto the medial layer [22].

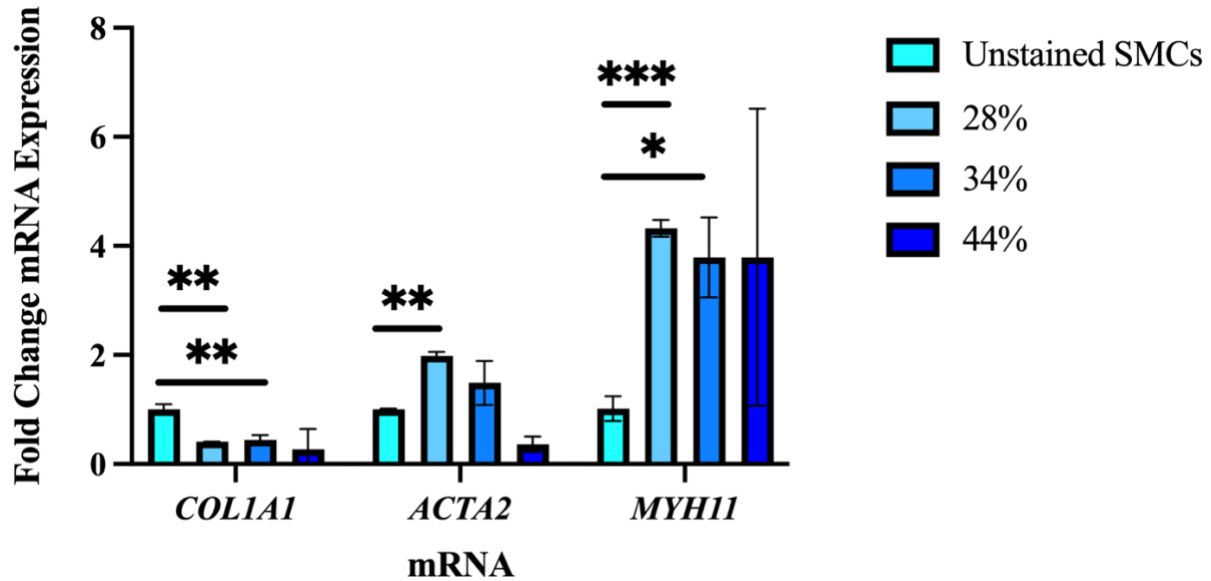


Figure 14: RT-PCR analysis of smooth muscle cell samples digested from TEBVs including an adventitial layer. The reference gene used was *RPLP0*. Genes of interest include *COL1A1*, *ACTA2*, and *MYH11*. Samples assessed were SMCs without CellTracker™ dye and monocultured in 2D, and SMCs cultured in 3D with an adventitial layer comprising 28%, 34%, and 44% of the total arterial wall thickness respectively. n = 3 technical replicates (1 biological replicate per sample group), *p < 0.05, **p < 0.01, ***p < 0.001.

For the samples containing an adventitia making up 28% of the total arterial wall thickness, a statistically significant fold-increase was seen in the *ACTA2* and *MYH11* genes, showing an increase in a contractile, mature phenotype in the smooth muscle cells. A statistically significant decrease in fold expression for collagen was observed for this sample, supplementing this conclusion. These results adhere to Technical Specification A3.

6. Discussion

Arterial fibrosis is a pathological remodeling process which stiffens arterial wall and thickens the arterial tunica adventitia, and it underlies a large majority of cardiovascular disease-related mortality. In vitro models of arterial environments can be implemented as tools for understanding the role of the adventitia in the development of arterial fibrosis and atherosclerosis, as well as evaluating the efficacies of potential therapies in the preclinical stage. In this report, I have designed, fabricated, and characterized the incorporation of a biomimetic tunica adventitia into a tissue-engineered blood vessel model.

6.1. Achievement of Technical Specifications

Table 5 lists the design specifications previously described for the development of the tunica adventitia and shows whether or not those specifications were achieved. Specifications listed in the table will be discussed in this section in order from top to bottom.

Table 7: Analysis of design specification achievement.

Specification Number	Specification	Expected Value	Measured Value
A1	Fibroblast cell viability	> 80% cell viability	> 80% viable
A2	Radial distance between the adventitial and medial layers	< 10 μm	< 10 μm
A3	Change in mRNA expression of smooth muscle cells	> 1.5-fold RNA expression increase of $\alpha\text{-SMA}$ and <i>MYH11</i>	1.8-4 fold increase in 28% & 34% samples (preliminary)
B1	Fibroblast initial cell concentration	250K-600K per 1.5 mm-diameter artery	250K-500K
B2	Collagen concentration of adventitial layer	0.5-2 mg/mL	5 mg/mL

B3	Adventitial layer thickness	30-40% of the arterial wall thickness	33-52%
B4	Adventitia reproducibility	Adventitia can be made in batches of 9, and each adventitia in the batch satisfies technical specifications B1-B3	4-10 adventitia per batch

The fabricated adventitial layers met Design-Independent Technical Specifications A1 and A2 and showed promising trends towards meeting A3. The MTS assay confirmed that the fibroblasts and smooth muscle cells embedded in the TEBV construct were more than 80% viable, ensuring that the cell populations remain viable during and after the fabrication process. The radial distance between the adventitial and medial layers was less than 10 μm , as confirmed by multi-photon microscopy of TEBV cross-sections. Greater than a 1.5-fold increase in the expression of ACTA2 and MYH11 was observed in the TEBV samples that had adventitia making up 28% and 34% of the total arterial wall, but the results were inconclusive for the sample with an adventitia making up 44% of the total arterial wall thickness. This may have been due to the small amount of RNA isolated from this sample group (see **Table 9**) despite this sample group containing comparable cell yields post-FACS as the 34% sample group (**Figure 13**). Regardless, these RT-PCR results are preliminary and would need to be repeated to confirm the observed trends.

The Design-Dependent Technical Specification B1 was met, with each adventitia fabricated containing between 250K-600K fibroblasts. Technical Specification B2 was not met, as the adventitial layer was fabricated using collagen sheets extruded at 5 mg/mL. The reason for this was three-fold: firstly, collagen sheets extruded within the physiological adventitial range of 0.5-2 mg/mL could either not extrude in a uniform, ultrathin collagen sheet, or if they could, they

could not be rolled into the adventitial layer without significant tears. Secondly, the mechanical properties collagen sheet extrusion process has been extensively characterized with 5 mg/mL collagen solutions [9]. Thirdly, the native adventitial layer contains substances other than collagen that were not incorporated into this model, such as elastin [30]. Since only collagen was used to build this adventitial model, the higher collagen concentration stands in place for the other substances found within the native layer.

Design-Dependent Technical Specification B3 was met by the sample group containing an adventitia that made up 34% of the total arterial wall thickness and was slightly out of the technical specification range for the 44% adventitial thickness sample. This discrepancy, as well as the discrepancy seen between the adventitial lengths measured between the 2 sample groups (**Figure 12**Error! Reference source not found.C), might be explained by the method of imaging the TEBV cross-section. TEBV constructs were placed on their side so that their cross-section was perpendicular to the microscope. This method of imaging may have significantly skewed the TEBV layer thicknesses measured, as described in **Section 6.3**. Cross-sectional imaging must be optimized and repeated to confirm that this Technical Specification can be consistently met and measured properly.

Design-Dependent Technical Specification B4 was not met, with 4-12 adventitia fabricated per batch. The number of adventitia made per batch varied on the size; 12 5-cm adventitia, 9 7.6-cm adventitia, and 6 11-cm adventitia were made per batch. Thus, while the 11-cm adventitia did not meet this specification, the 7.6-cm and 5-cm adventitia met and exceeded this technical specification. Each adventitia in the batch consistently met the rest of the Design-Dependent Technical Specifications B1-B3.

6.2. Comparison to Previous Models

As described in **Sections 2.2 and 2.3**, there have yet to be significant efforts to engineer the tunica adventitia towards building a more complex tissue-engineered blood vessel (TEBV) model. As previously described, Patel et al. were able to engineer self-forming adventitial rings that, when stacked, formed an adventitial vessel. However, (1) they were not able to handle a thin adventitial layer within the percent thickness reported here due to the fragility of their fabrication method, and (2) the adventitial vessel was not incorporated into a vessel model containing a medial or intimal layer to capture the physiology of native arteries more effectively. In contrast, the adventitial layer made in this project were able to remain as a thin but intact layer due to the rolling method employed, and the adventitial layer was added to a blood vessel platform containing the medial layer and that is currently optimizing the addition of the intimal layer. Thus, the work done in this project to fabricate the adventitial layer alongside the medial and intimal TEBV layer improve upon the existing simplified fibrosis models discussed in **Table 1** and adventitial engineering methods to create a more complex platform to study arterial disease.

6.3. Project Limitations

Fungal & Bacterial Contamination

Contamination was a consistent inhibitor of project progress throughout the year, both at the 2D flask and 3D TEBV level. At the 2D level, we attempted to avoid fungal and bacteria contaminations by adding 1% Pen/Strep to smooth muscle cell (SMC) and fibroblast (FB) media, and 1% Fungin to each flask after changing media. This addition of antibacterial and antifungal reagents likely contributed to the slowed growth rate we observed in the SMC and FB cell cultures. Regardless of this addition, as well as thoroughly cleaning the tissue culture hood and incubator used to culture these cells, 2D flasks still got contaminated. The single tissue culture

hood and incubator the Chaikof Lab has for this cell culture is frequently used by several researchers for a variety of applications, including bacterial culture and other culture methods that involve lifting the sash above the recommended height to maintain sterility. In addition, it is possible that the bacterial and fungal infections observed in these flasks were immune to the antibacterial and antifungal reagents used in the cell cultures.

Moreover, the TEBV constructs themselves would very frequently get contaminated, reducing the batch replicate number and sometimes killing the entire batch. It was extremely difficult to maintain sterility throughout the TEBV rolling process because the mechanical parts used to roll the collagen sheets into TEBVs are massive and have to be stored outside of the tissue culture hood on a benchtop to ensure adequate space and air flow through the hood for other users. To maintain sterility, the necessary tools were autoclaved the collagen sheets themselves, as well as the parts that could not be autoclaved, were sterilized under UV light in the tissue culture hood for at least 30 minutes. Despite these efforts, at least 2 TEBV constructs got contaminated in every batch.

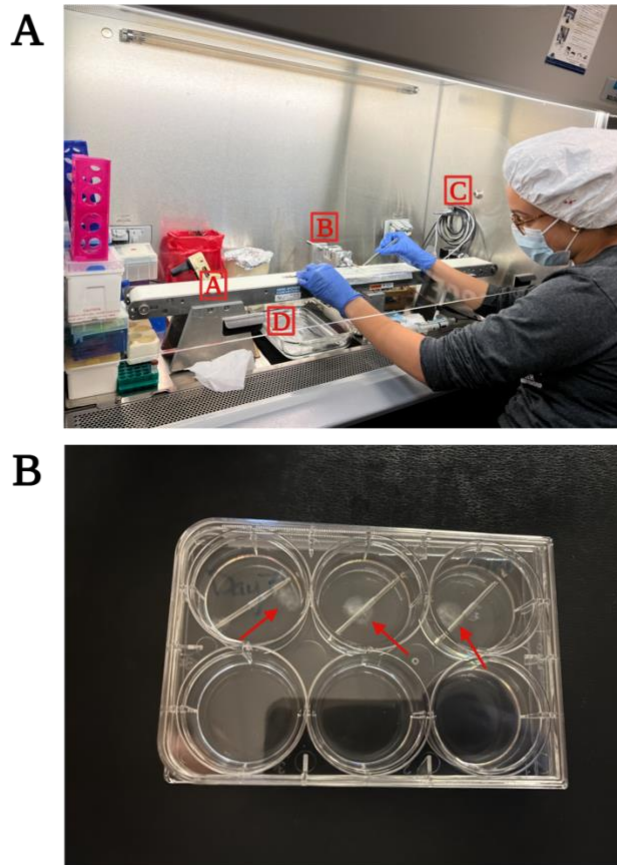


Figure 15: Setup of tissue-engineered blood vessel (TEBV) rolling process leading to contamination. (A) TEBV rolling setup in the tissue hood: [A] Conveyor belt that moves collagen sheet forward towards mandrel rotator. [B] Mandrel rotator that wraps TEBV around the split mandrel. [C] Cord setup that connects [A] and [B] to power. [D] Collagen sheets in Hanks Balanced Salt Solution. [A]-[D] are sterilized under UV radiation for at least 30 minutes, and collagen sheets are sterilized in a 1% Fungin solution. (B) Fungal contamination of TEBVs two days after rolling.

TEBV Fabrication Timescale

Another limiting factor of this project was the TEBV fabrication timescale. Fabrication took 2 weeks per batch before the constructs could be analyzed. The first week of fabrication involved collagen sheet extrusion, spreading collagen sheets onto glass slides, and seeding cell solutions on the collagen sheets. The cell-laden sheets would culture over the weekend, then the sheets would be rolled into TEBVs. After 7 days of static culture, the TEBVs would be ready for

data collection. For this reason, it became difficult to achieve a high replicate number per batch, and we were unable to fabricate a large number of batches over the year.

In addition, the length of each step for TEBV fabrication became a limiting factor for the number of replicates made per batch. The Chaikof Lab has the resources to make 10 TEBVs per batch due to material constraints. Even so, extruding enough collagen for 10 TEBVs plus the adventitial layers became a difficult task because of how long this technical process takes.

Imaging of the TEBV Cross-Section

As mentioned in **Section 4.3.2**, the method of imaging the tissue-engineered blood vessel (TEBV) cross-section must be further optimized to accurately assess TEBV layer thickness.

Figure 16 shows a stitched image of a TEBV lumen taken using multi-photon microscopy. On the left-hand side of the vessel wall, the adventitial layer (yellow) completely covers the inner medial layer (red), but clear layer separation is observed on the right-hand side of the image. This is likely caused by the TEBV not being perfectly perpendicular to the microscope during imaging; in other words, the TEBV was slightly angled with respect to the microscope, and the resulting captured image reflects this by the adventitial layer appearing much thicker on one side than the other.

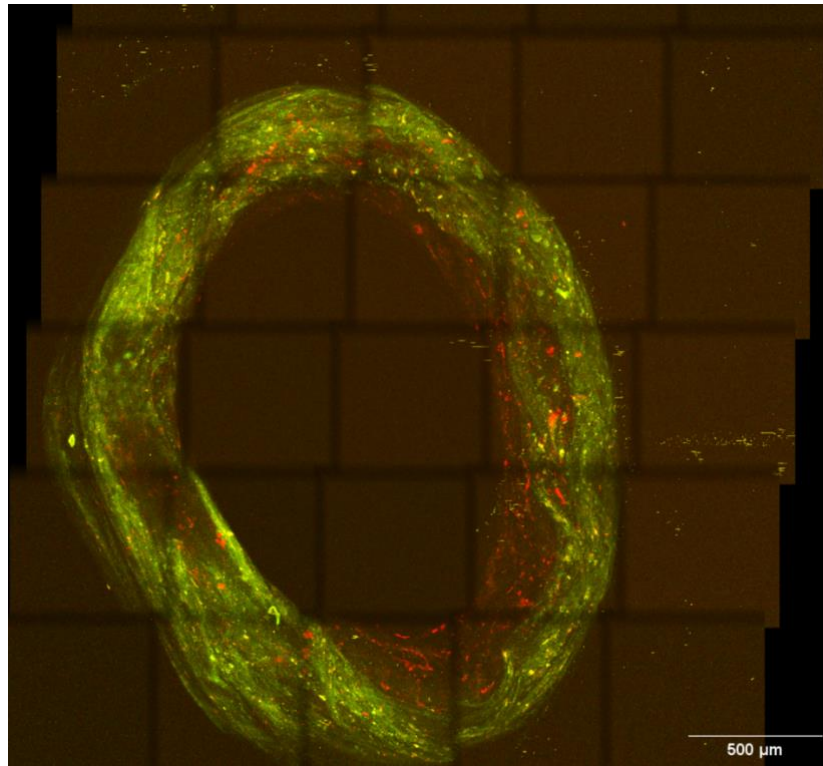


Figure 16: Stitched cross-sectional image of a TEBV. In this image, the adventitial layer (yellow) nearly completely covers the medial layer (red) on the left-hand side of the vessel wall, but much clearer separation between the layers is observed on the right-hand side of the vessel wall.

So far, two methods to remedy this problem have been explored. Firstly, attempts to cut small sections of the TEBV with a razor blade for greater ease of imaging have proved unsuccessful. The jagged edge left by the razor blade makes it difficult to achieve a clear, representative image of the TEBV cross section and layer thickness. Secondly, the Chaikof Lab has been working with the Histology Core at Beth Israel Deaconess Medical Center (BIDMC) to use paraffin sectioning or cryo-sectioning for cross-sectional imaging. However, so far these methods have resulted in poor layer-to-layer apposition, making it very difficult to capture a representative image of the TEBV cross section without severe layer delamination. The sectioning method is currently being optimized in the Chaikof Lab as a promising avenue for further multi-photon imaging. As an optimized imaging method is being developed, it will be

important to refer to engineering standards for interpreting images of polymeric tissue scaffolds (ASTM F2603-06, **Appendix 7.3.3**).

6.4. Future Work: Modeling Fibrosis

The ultimate goal of this project is to fabricate a tri-layered tissue-engineered blood vessel to use to study fibrosis in vitro. With this in mind, it is important to consider what factors should be taken into consideration when these TEBVs are used as a fibrotic model that would influence the design-dependent and design-independent technical specifications for effectively modelling fibrosis.

6.4.1. Modeling and Characterizing Fibrosis

Potential Modeling Considerations:

- Introduce cytokines into flow. It may be easiest and most effective to simply introduce an inflammatory factor, such as TGF- β , into the flow stream through the perfused TEBV. This growth factor would transition FBs to active MyoFBs, and the continuous perfusion of TGF- β may be sufficient to create a fibrotic model.
- Pattern adventitial layer with cytokines for release. It may be more fruitful to transition FBs into active MyoFBs sooner, even before the adventitial layer is created. TGF- β could be added to the collagen-FB solution used to make the adventitial layer so that the FBs are directly exposed to the growth factor immediately.
- Mechanical stress that would induce MyoFB differentiation. Along with biochemical induction of MyoFB activation, activation can be induced via mechanical stimuli. Mechanical stress and strain, or mechanically creating vessel injury, are important factors in activating MyoFBs [31]. It may be simpler and more cost-effective to apply minimal, continuous strain to the TEBV to create wound injury, modeling fibrosis.

Potential Characterization Considerations:

- The effects of mechanical and biochemical stimuli on other cell types in the TEBV. These biochemical and mechanical stimuli will have an effect on the ECs and SMCs embedded in the TEBV. It will be important to quantify these effects to ensure that fibrosis is accurately modeled in all vessel layers.
- Maintaining stimuli over time to continuously promote MyoFB activation and differentiation. It will be critical to assess how long-term exposure to biochemical agents and mechanical stimuli affects the integrity of the model. For example, excessive mechanical stimuli may be more harmful to the overall structural integrity of the vessel, breaking down the model over time. It will be important to consider the longer-term effects of stimuli exposure to optimize both fibrosis development but also the integrity of the TEBV.

When the fabrication of tri-layered TEBVs is streamlined, they will be used to model fibrosis *in vitro*. The process of modeling fibrosis must achieve certain design-independent technical specifications to measure and verify that fibrosis is effectively induced in the arterial environments. Moreover, these technical specifications influenced the scope of the technical specifications used in this capstone project to fabricate and characterize the adventitia layer. These fibrosis induction-related technical specifications can be found in **Appendix 7.2**.

6.4.2. Preliminary Work: Sorting Fibroblasts, Smooth Muscle Cells, & Myofibroblasts

A critical aspect of characterizing fibrosis induced in the tissue-engineered blood vessel (TEBV) model would include effectively separating fibroblasts (FBs), smooth muscle cells (SMCs), and myofibroblasts (MyoFBs). This step would be important for Technical Specification A6, but it would also inform the development and progression of fibrosis in the TEBV model. To begin this work, cell sorting analysis was employed to assess what

combination of antibodies would sort these three cell populations for further gene expression analysis.

Experimental Setup. SMCs and FBs were seeded into 6-well plates at 250K per well. Three groups were used for this experiment: a SMC monoculture, a FB monoculture, and a SMC-FB co-culture. On each plate, 3 wells were cultured without TGF- β and cultured for 6 days. The other 3 wells were cultured in the absence of TGF- β for 3 days, then spiked with 10 ng/mL TGF- β (Pepro Tech) and cultured for 3 more days. All cells were cultured in Stromal Cell Growth Media (Lonza).

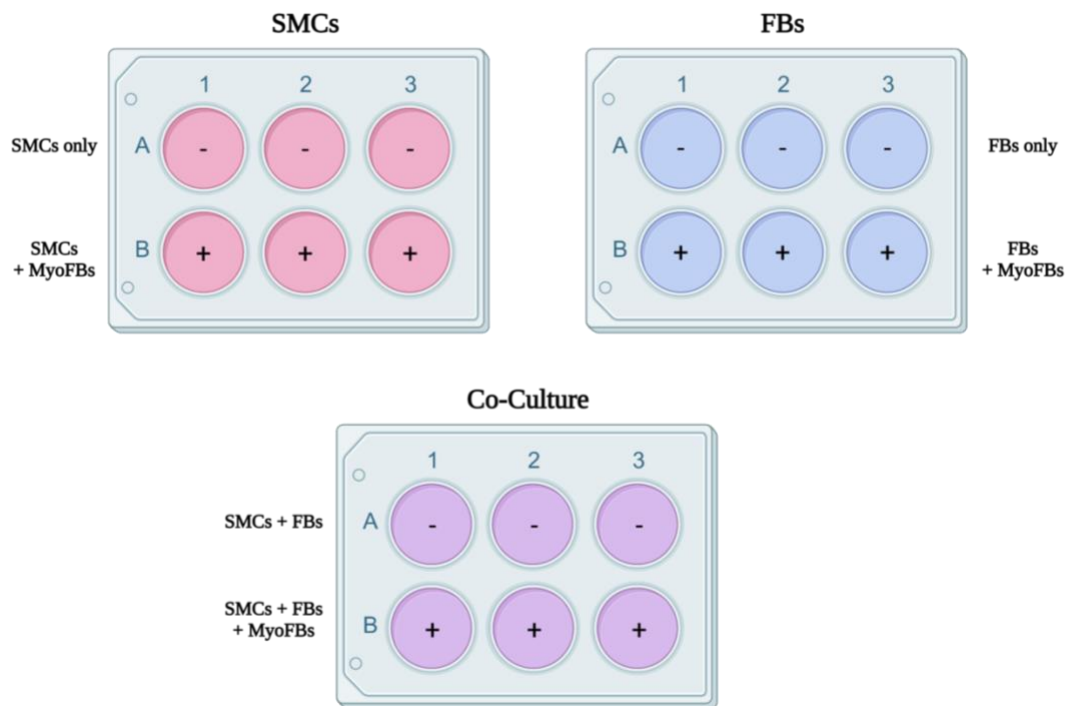


Figure 17: Experimental Setup of TGF- β exposure to SMCs and FBs. SMCs only, FBs only, and a co-culture of SMCs and FBs were cultured with and without TGF- β for 6 days. n = 3 biological replicates.

After 6 days of culture, cells were trypsinized and resuspended in 0.3 mL PBS for antibody tagging and cell sorting analysis. Previous literature suggests that both SMCs and FBs

transition to a MyoFB cell state when activated by TGF- β , so it was hypothesized that MyoFBs would be in each TGF- β well.

Cell Sorting Analysis. Previous studies have shown that CD90 is an effective antibody that binds specifically to FBs to sort SMCs from FBs [32]. As a preliminary test, CD90 (BD Biosciences) and CD140b (Sigma Aldrich), a marker shared by both FBs and SMCs, were attached to the cell types as described in the protocol [33]. Samples were sorted using the Cytoflex SRT instrument. A forward scatter threshold was used on the populations to gate live, single cells. The gating strategy can be found in **Appendix 7.2**.

Results. FBs, MyoFBs, and SMCs were sorted. The results were analyzed to determine whether SMC, FB, and MyoFB populations could be distinctly identified. As shown in **Figure 18**, there is not a substantial shift in CD90^{pos} cells between the TGF- β^+ and TGF- β^- samples; thus, CD90 cannot be used as a marker to differentiate between FB and MyoFB populations. Conversely, there is a shift that can be gated between the CD140b^{pos}, TGF- β^- sample and CD140b^{pos}, TGF- β^+ sample. This makes CD140b a promising candidate for separating FBs from MyoFB populations. However, the antibodies were inconclusive for sorting any other combination of cell types (see the density plots in **Appendix 7.2, Figure 25-Figure 27**).

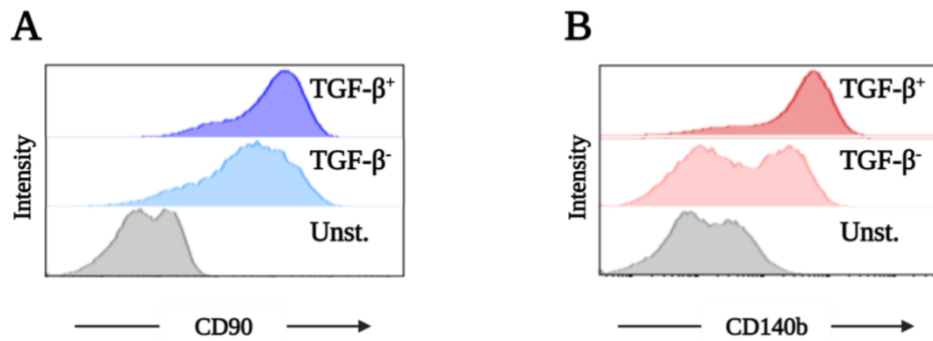


Figure 18: CD 90 & CD140b FACS histograms of FB only samples with and without TGF- β exposure. (A) Histograms for CD90-tagged FBs spiked with TGF- β (TGF- β^+) and cultured without TGF- β (TGF- β^-), and FBs cultured without TGF- β and untagged as a control (Unst.). (B) Histograms for CD140b-tagged FBs spiked with TGF- β (TGF- β^+) and cultured without TGF- β (TGF- β^-), and FBs cultured without TGF- β and untagged as a control (Unst.), $n = 3$ biological replicated for each sample group.

Future Work. More antibodies must be tested to use a combination that effectively sorts FBs, SMCs, and MyoFBs from each other. While the CD90 antibody proved inconclusive for sorting FBs from SMCs, this experiment must be repeated to confirm this. In addition, CellTracker™ dyes can be used to sort FBs from SMCs, and another antibody can be used to sort out MyoFBs. Recently, Turley et al. have used LRRC15 to sort specifically for cancer-associated fibroblasts (CAFs), a diseased fibroblast type similar to MyoFBs [34]. This could be a promising antibody candidate for sorting MyoFBs from SMCs.

6.5. Project Impact

The tissue-engineered tunica adventitia is an important component of the physiologically relevant tissue-engineered blood vessel model that can have an important role in modeling fibrosis in a complex 3D blood vessel microenvironment and informing the preclinical screening of anti-fibrotic drug candidates. The adventitial parameter optimization done within the scope of this project and the novel fabrication method used to engineer the adventitia make it an improved *in vitro* platform than existing models towards better recapitulating vascular complexity and understanding the development of arterial fibrosis, atherosclerosis, and ultimately cardiovascular disease. In addition, there is potential for the tri-layered TEBV to be used as engineered vascular grafts, since it mimics the cellular and collagen fiber alignment found in native arteries. Ultimately, the engineered tunica adventitia and tri-layered TEBV serve as promising tools for modeling arterial disease and other tissue engineering applications.

References

- [1] K. Mc Namara, H. Alzubaidi, and J. K. Jackson, “Cardiovascular disease as a leading cause of death: how are pharmacists getting involved?,” *Integr. Pharm. Res. Pract.*, vol. 8, pp. 1–11, Feb. 2019, doi: 10.2147/IPRP.S133088.
- [2] W. Jiang, Y. Xiong, X. Li, and Y. Yang, “Cardiac Fibrosis: Cellular Effectors, Molecular Pathways, and Exosomal Roles,” *Front. Cardiovasc. Med.*, vol. 8, 2021, Accessed: Sep. 05, 2022. [Online]. Available: <https://www.frontiersin.org/articles/10.3389/fcvm.2021.715258>
- [3] A. A. Mercadante and A. Raja, “Anatomy, Arteries,” in *StatPearls*, Treasure Island (FL): StatPearls Publishing, 2022. Accessed: Sep. 06, 2022. [Online]. Available: <http://www.ncbi.nlm.nih.gov/books/NBK547743/>
- [4] “Fibroblasts and myofibroblasts in wound healing - PMC.” <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4226391/> (accessed Sep. 04, 2022).
- [5] F. Klingberg, B. Hinz, and E. S. White, “The myofibroblast matrix: implications for tissue repair and fibrosis,” *J. Pathol.*, vol. 229, no. 2, pp. 298–309, Jan. 2013, doi: 10.1002/path.4104.
- [6] E. Balza, L. Borsi, G. Allemanni, and L. Zardi, “Transforming growth factor β regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts,” *FEBS Lett.*, vol. 228, no. 1, pp. 42–44, 1988, doi: 10.1016/0014-5793(88)80580-5.
- [7] J. T. Kuwabara and M. D. Tallquist, “Tracking Adventitial Fibroblast Contribution to Disease,” *Arterioscler. Thromb. Vasc. Biol.*, vol. 37, no. 9, pp. 1598–1607, Sep. 2017, doi: 10.1161/ATVBAHA.117.308199.
- [8] X. Zhang *et al.*, “Modeling early stage atherosclerosis in a primary human vascular microphysiological system,” *Nat. Commun.*, vol. 11, no. 1, Art. no. 1, Oct. 2020, doi: 10.1038/s41467-020-19197-8.
- [9] “Continuous Formation of Ultrathin, Strong Collagen Sheets with Tunable Anisotropy and Compaction | ACS Biomaterials Science & Engineering.” <https://pubs.acs.org/doi/10.1021/acsbiomaterials.0c00321?cookieSet=1> (accessed Sep. 03, 2022).
- [10] M. Carrabba and P. Madeddu, “Current Strategies for the Manufacture of Small Size Tissue Engineering Vascular Grafts,” *Front. Bioeng. Biotechnol.*, vol. 6, 2018, Accessed: Sep. 05, 2022. [Online]. Available: <https://www.frontiersin.org/articles/10.3389/fbioe.2018.00041>
- [11] B. Patel, Z. Xu, C. B. Pinnock, L. S. Kabbani, and M. T. Lam, “Self-assembled Collagen-Fibrin Hydrogel Reinforces Tissue Engineered Adventitia Vessels Seeded with Human Fibroblasts,” *Sci. Rep.*, vol. 8, no. 1, Art. no. 1, Feb. 2018, doi: 10.1038/s41598-018-21681-7.
- [12] M. Mirbagheri, V. Adibnia, B. R. Hughes, S. D. Waldman, X. Banquy, and D. K. Hwang, “Advanced cell culture platforms: a growing quest for emulating natural tissues,” *Mater. Horiz.*, vol. 6, no. 1, pp. 45–71, Jan. 2019, doi: 10.1039/C8MH00803E.
- [13] A. P. Epa *et al.*, “Normal Human Lung Epithelial Cells Inhibit Transforming Growth Factor- β Induced Myofibroblast Differentiation via Prostaglandin E2,” *PLOS ONE*, vol. 10, no. 8, p. e0135266, Aug. 2015, doi: 10.1371/journal.pone.0135266.
- [14] G. A. Figtree, K. J. Bubb, O. Tang, E. Kizana, and C. Gentile, “Vascularized Cardiac Spheroids as Novel 3D in vitro Models to Study Cardiac Fibrosis,” *Cells Tissues Organs*, vol. 204, no. 3–4, pp. 191–198, 2017, doi: 10.1159/000477436.

- [15] K. P. Kanebratt *et al.*, “Primary Human Hepatocyte Spheroid Model as a 3D In Vitro Platform for Metabolism Studies,” *J. Pharm. Sci.*, vol. 110, no. 1, pp. 422–431, Jan. 2021, doi: 10.1016/j.xphs.2020.10.043.
- [16] X. Ma *et al.*, “3D bioprinting of functional tissue models for personalized drug screening and in vitro disease modeling,” *Adv. Drug Deliv. Rev.*, vol. 132, pp. 235–251, Jul. 2018, doi: 10.1016/j.addr.2018.06.011.
- [17] L. M. Norona, D. G. Nguyen, D. A. Gerber, S. C. Presnell, and E. L. LeCluyse, “Editor’s Highlight: Modeling Compound-Induced Fibrogenesis In Vitro Using Three-Dimensional Bioprinted Human Liver Tissues,” *Toxicol. Sci.*, vol. 154, no. 2, pp. 354–367, Dec. 2016, doi: 10.1093/toxsci/kfw169.
- [18] M. Asmani *et al.*, “Fibrotic microtissue array to predict anti-fibrosis drug efficacy,” *Nat. Commun.*, vol. 9, no. 1, Art. no. 1, May 2018, doi: 10.1038/s41467-018-04336-z.
- [19] S. Alimperti *et al.*, “Three-dimensional biomimetic vascular model reveals a RhoA, Rac1, and N-cadherin balance in mural cell–endothelial cell-regulated barrier function,” *Proc. Natl. Acad. Sci.*, vol. 114, no. 33, pp. 8758–8763, Aug. 2017, doi: 10.1073/pnas.1618333114.
- [20] O. Mastikhina *et al.*, “Human cardiac fibrosis-on-a-chip model recapitulates disease hallmarks and can serve as a platform for drug testing,” *Biomaterials*, vol. 233, p. 119741, Mar. 2020, doi: 10.1016/j.biomaterials.2019.119741.
- [21] A. Sundarakrishnan *et al.*, “Bioengineered in Vitro Tissue Model of Fibroblast Activation for Modeling Pulmonary Fibrosis,” *ACS Biomater. Sci. Eng.*, vol. 5, no. 5, pp. 2417–2429, May 2019, doi: 10.1021/acsbiomaterials.8b01262.
- [22] G. K. Owens, M. S. Kumar, and B. R. Wamhoff, “Molecular Regulation of Vascular Smooth Muscle Cell Differentiation in Development and Disease,” *Physiol. Rev.*, vol. 84, no. 3, pp. 767–801, Jul. 2004, doi: 10.1152/physrev.00041.2003.
- [23] C. E. Fernandez *et al.*, “Human Vascular Microphysiological System for in vitro Drug Screening,” *Sci. Rep.*, vol. 6, no. 1, Art. no. 1, Feb. 2016, doi: 10.1038/srep21579.
- [24] K. Witter, Z. Tonar, and H. Schöpper, “How many Layers has the Adventitia? – Structure of the Arterial Tunica Externa Revisited,” *Anat. Histol. Embryol.*, vol. 46, no. 2, pp. 110–120, 2017, doi: 10.1111/ahe.12239.
- [25] G. D. Pins, D. L. Christiansen, R. Patel, and F. H. Silver, “Self-assembly of collagen fibers. Influence of fibrillar alignment and decorin on mechanical properties,” *Biophys. J.*, vol. 73, no. 4, pp. 2164–2172, Oct. 1997, doi: 10.1016/S0006-3495(97)78247-X.
- [26] T. Deng *et al.*, “HMGN1 Modulates Nucleosome Occupancy and DNase I Hypersensitivity at the CpG Island Promoters of Embryonic Stem Cells,” *Mol. Cell. Biol.*, vol. 33, no. 16, pp. 3377–3389, Aug. 2013, doi: 10.1128/MCB.00435-13.
- [27] “RNeasy Mini Handbook - (EN) - QIAGEN.” <https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en> (accessed Mar. 29, 2023).
- [28] “maxima_hminus_mastermix.pdf.” Accessed: Mar. 29, 2023. [Online]. Available: https://assets.fishersci.com/TFS-Assets/LSG/manuals/maxima_hminus_mastermix.pdf
- [29] “4442661C.pdf.” Accessed: Mar. 29, 2023. [Online]. Available: <https://tools.thermofisher.com/content/sfs/manuals/4442661C.pdf>
- [30] J. M. Hunt and B. B. Graham, “Pulmonary Hypertension/Pulmonary Arterial Hypertension,” in *Pathobiology of Human Disease*, L. M. McManus and R. N. Mitchell,

- Eds., San Diego: Academic Press, 2014, pp. 2625–2635. doi: 10.1016/B978-0-12-386456-7.05306-5.
- [31] A. K. Schroer and W. D. Merryman, “Mechanobiology of myofibroblast adhesion in fibrotic cardiac disease,” *J. Cell Sci.*, vol. 128, no. 10, pp. 1865–1875, May 2015, doi: 10.1242/jcs.162891.
 - [32] S. C. Weber *et al.*, “Isolation and Culture of Fibroblasts, Vascular Smooth Muscle, and Endothelial Cells From the Fetal Rat Ductus Arteriosus,” *Pediatr. Res.*, vol. 70, no. 3, Art. no. 3, Sep. 2011, doi: 10.1203/PDR.0b013e318225f748.
 - [33] K. Holmes, L. M. Lantz, B. J. Fowlkes, I. Schmid, and J. V. Giorgi, “Preparation of Cells and Reagents for Flow Cytometry,” *Curr. Protoc. Immunol.*, vol. 44, no. 1, Aug. 2001, doi: 10.1002/0471142735.im0503s44.
 - [34] A. T. Krishnamurty *et al.*, “LRRC15⁺ myofibroblasts dictate the stromal setpoint to suppress tumour immunity,” *Nature*, vol. 611, no. 7934, pp. 148–154, Nov. 2022, doi: 10.1038/s41586-022-05272-1.
 - [35] J. C. Kohn, M. C. Lampi, and C. A. Reinhart-King, “Age-related vascular stiffening: causes and consequences,” *Front. Genet.*, vol. 6, 2015, Accessed: Oct. 11, 2022. [Online]. Available: <https://www.frontiersin.org/articles/10.3389/fgene.2015.00112>
 - [36] J. P. J. Halcox *et al.*, “Prognostic Value of Coronary Vascular Endothelial Dysfunction,” *Circulation*, vol. 106, no. 6, pp. 653–658, Aug. 2002, doi: 10.1161/01.CIR.0000025404.78001.D8.
 - [37] “Aortic carboxypeptidase-like protein regulates vascular adventitial progenitor and fibroblast differentiation through myocardin related transcription factor A | Scientific Reports.” <https://www.nature.com/articles/s41598-021-82941-7> (accessed Oct. 15, 2022).
 - [38] “Standard Practice for Quantifying Cell Proliferation in 3D Scaffolds by a Nondestructive Method.” <https://www.astm.org/f3504-21.html> (accessed Mar. 30, 2023).
 - [39] “ASTM F3510-21 - Standard Guide for Characterizing Fiber-Based Constructs for Tissue-Engineered Medical Products.” <https://webstore.ansi.org/standards/astm/astmf351021> (accessed Mar. 30, 2023).
 - [40] “Standard Guide for Interpreting Images of Polymeric Tissue Scaffolds.” <https://www.astm.org/f2603-06r20.html> (accessed Mar. 30, 2023).

7. Appendix

7.1. FACS & RT-PCR Data

Table 8: Data for cell yield before and after FACS.

Sample	Number of Cells Seeded	Number of Cells Retrieved from FACS	Percent Ratio (%)
28% - FBs	345,000	2360	0.68
28% - SMCs	1,380,000	41,438	3.00
34% - FBs	460,000	51,528	11.20
34% - SMCs	1,380,000	186,068	13.48
44% - FBs	690,000	52,256	7.57
44% - SMCs	1,380,000	158,796	11.51

Table 9: Isolated RNA concentrations and purity ratios for FACS samples.

Sample	Isolated RNA (ng/μL)	A260/A280	A260/A230
Unstained SMCs (Control)	165.5	2.07	1.65
Unstained FBs (Control)	66.7	2.10	0.38
28% SMCs	6.5	1.62	0.38
28% FBs	18.7	1.55	0.25
34% SMCs	4.2	2.03	0.48
34% FBs	2.0	1.87	0.06
44% SMCs	0.3	-5.55	0.00
44% FBs	0.6	4.18	0.03

Gating Strategy

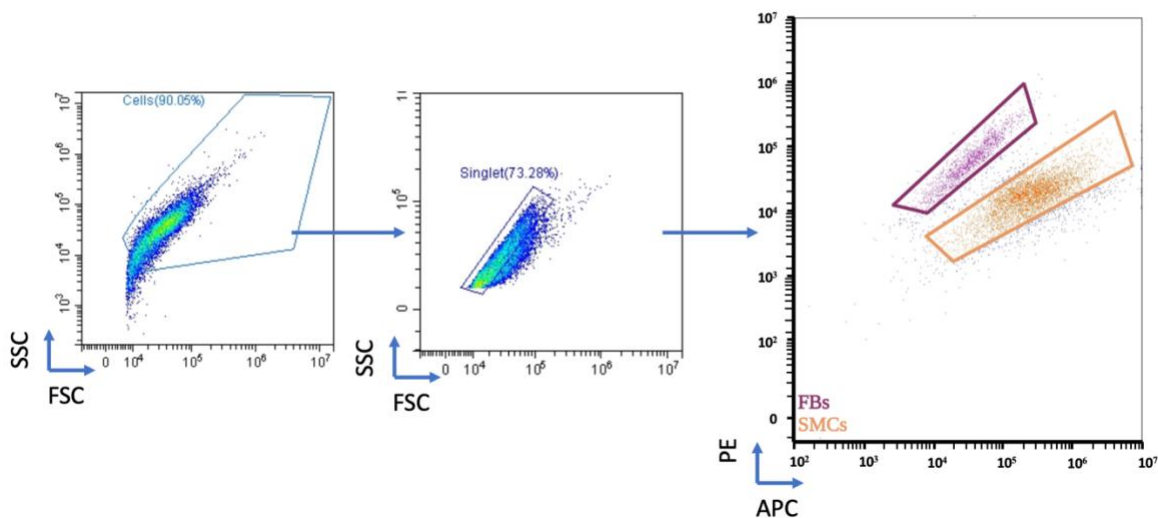


Figure 19: Gating strategy for FB & SMC FACS. First, a forward scatter threshold was applied to gate live cells (left), then another threshold was applied to gate single cells (middle). Finally, gates were used to separate fibroblasts (purple) from smooth muscle cells (orange).

RT-PCR Amplification Curves

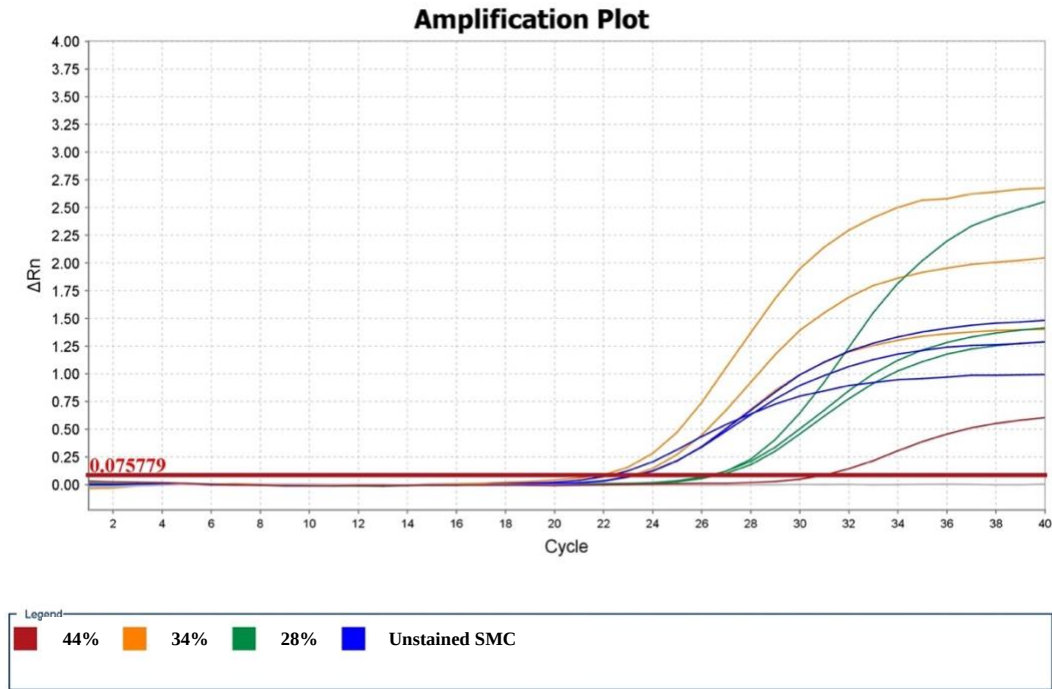


Figure 20: *RPLP0* amplification curve. The legend percent labels refer to the percent adventitial thickness of the total arterial wall. Unstained SMCs are the 2D smooth muscle cell control.

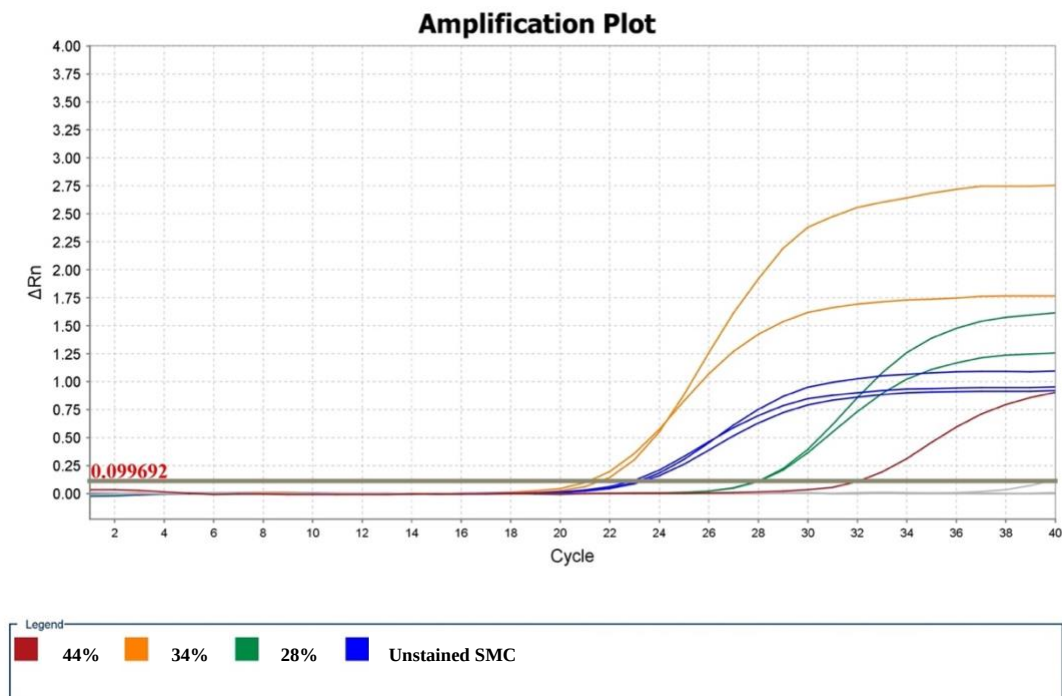


Figure 21: *COL1A1* amplification curve. The legend percent labels refer to the percent adventitial thickness of the total arterial wall. Unstained SMCs are the 2D smooth muscle cell control.

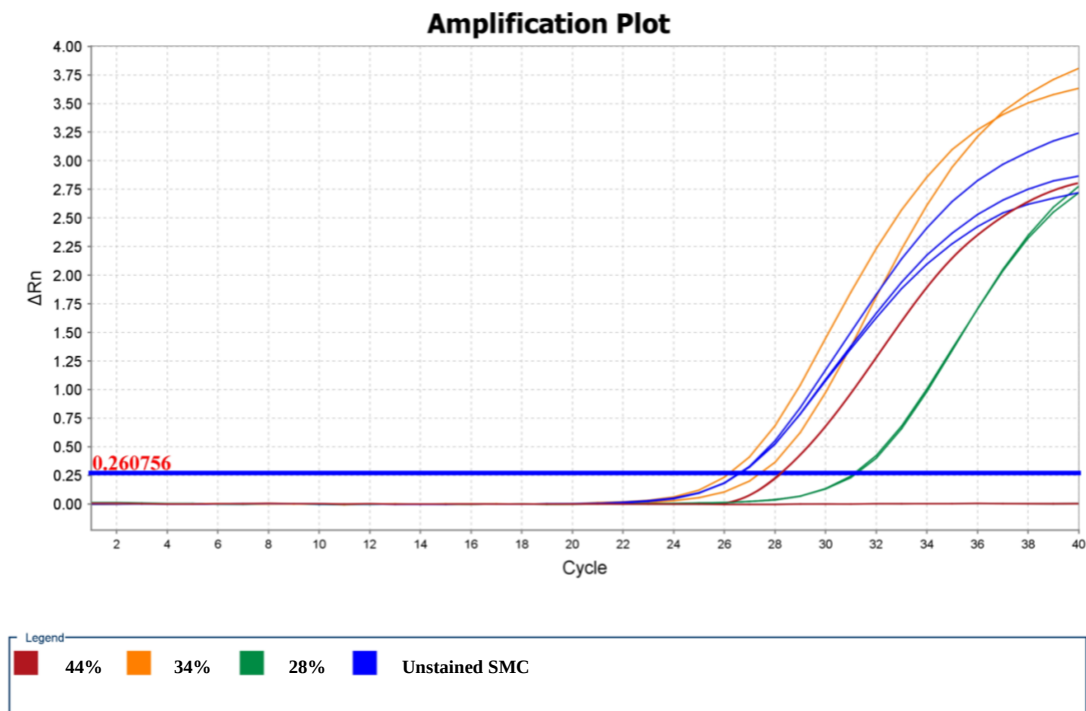


Figure 22: ACTA2 amplification curve. The legend percent labels refer to the percent adventitial thickness of the total arterial wall. Unstained SMCs are the 2D smooth muscle cell control.

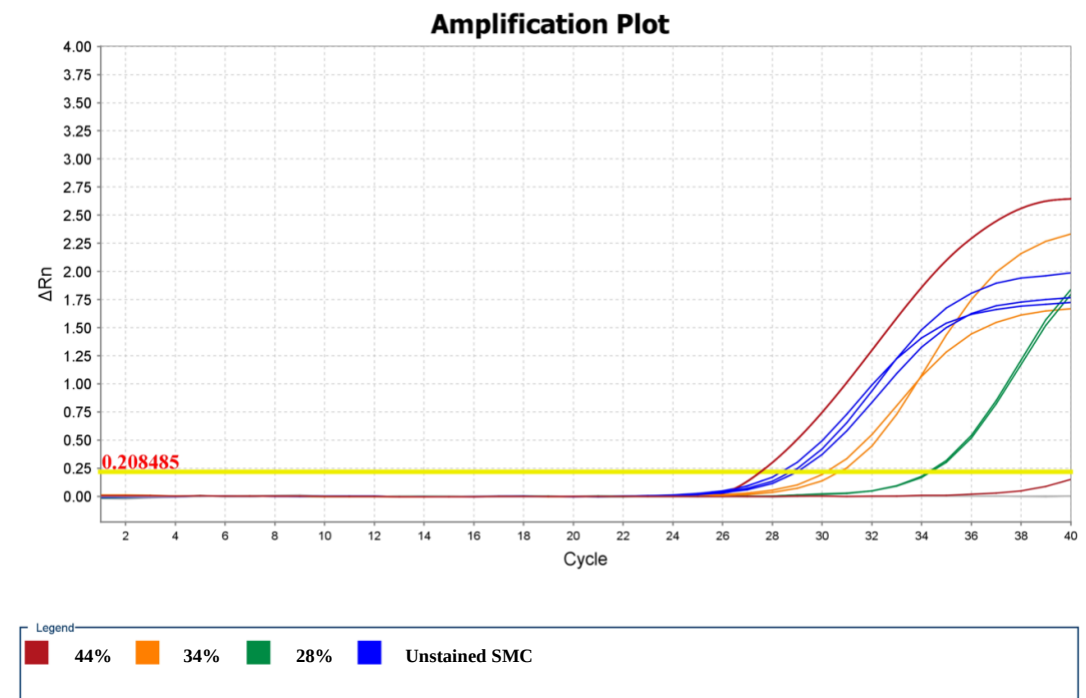


Figure 23: MYH11 amplification curve. The legend percent labels refer to the percent adventitial thickness of the total arterial wall. Unstained SMCs are the 2D smooth muscle cell control.

7.2. Modeling Fibrosis

Design-Independent Technical Specifications for Modeling Fibrosis

Table 10: Potential Design-Independent Technical Specifications & Measurement Methods for Modeling Fibrosis.

Specification Number	Specification	Measurement Method
A1	Adventitial layer thickness must be between 20-25% of the arterial wall thickness post-TGF- β exposure	Analysis of multi-photon images using second-harmonic imaging of collagen
A2	Arterial stiffness must increase by at least 50% following TGF- β exposure	Longitudinal tensile strain testing pre- and post-TGF- β exposure
A3	Phenylephrine-induced vasoconstriction must be reduced by at least 50% following TGF- β exposure	1uM phenylephrine added to flow after one week of perfusion culture; measure vessel diameter pre-and post-TGF- β exposure using (image analysis?)
A4	Caffeine-induced vasodilation must be reduced by at least 50% following TGF- β exposure	Caffeine doses ranging from 10^{-9} to 10^{-2} M will be perfused through the TEBVs, and image analysis will be used to assess the dose-dependent responses
A5	Arterial wall collagen expression must increase by at least 50% following TGF- β exposure	Analysis of multi-photon image color intensity using second-harmonic imaging of collagen
A6	Fibroblasts and smooth muscle cells that have transitioned to myofibroblasts must show at least a 1.5-fold RNA expression increase of α -SMA, calponin, and SM-22 post-TGF- β exposure	RT-PCR analysis of α -SMA and calponin markers before and after TGF- β exposure

Justifications

A1: Change in Adventitial Layer Thickness

In arterial hypertension, the adventitial layer of small-diameter arteries increases to 20-25% of the vessel wall thickness [30]. As activated myofibroblasts (MyoFBs) deposit collagen, the collagen fibrils within the layer become more and more dense which increases the entire layer's thickness.

A2: Change in Artery Wall Stiffness

Healthy human arteries have an elastic modulus of 1.5 MPa, which increases to 2.3-3.7 MPa in atherosclerotic arteries [35]. Due to the increase in collagen fibril density due to activated MyoFBs depositing excess collagen, the stiffness of the adventitial and medial layers increases. This increase has been documented for atherosclerotic arteries, which undergo a comparable stiffening due to excess collagen deposition.

A3 & A4: Change in Artery Vasoconstriction & Vasodilation

Artery constriction and dilation should mimic vasoconstriction and vasodilation values for existing TEBVs that are used for disease modeling [23]. Moreover, the change in artery constriction and dilation should be proportional to the change in coronary blood flow observed between healthy and diseased arteries, which has been reported to be 50-75% [36]. As the artery wall becomes stiffer and more collagen-dense, it should be more restricted to vasoconstriction and vasodilation compared to a pre-fibrotic artery.

A5: Change in Artery Wall Collagen Expression

As previously mentioned, collagen expression in fibrotic arteries should increase due to the excess MyoFB collagen deposition. The change in collagen expression will likely be

proportional to arterial wall stiffness; the stiffer the artery wall becomes, the more collagen is being deposited.

A6: Change in Fibroblast Cell Marker Expression

When FBs and SMCs transition to MyoFBs, they gene expression of α -SMA, calponin, and SM-22 increases 1.5-3 times [23][37]. These cellular markers will be quantified to assess whether FBs and SMCs have successfully transitioned to MyoFBs, which is a necessary step to achieve a fibrotic TEBV.

Preliminary Data of Cell Sorting Smooth Muscle Cells, Fibroblasts, and Myofibroblasts

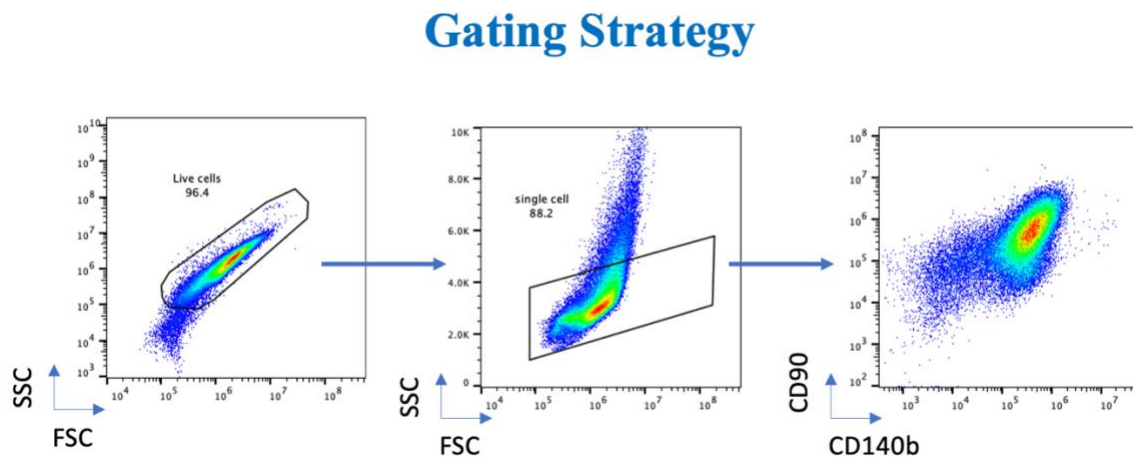


Figure 24: Gating strategy for SMC, FB, and MyoFB sorting. A forward scatter threshold was applied to gate live cells (left), then another threshold was applied to gate single cells (middle). These gates allowed for the capture of live, single cells tagged with CD90 and/or CD140b (right).

SMCs

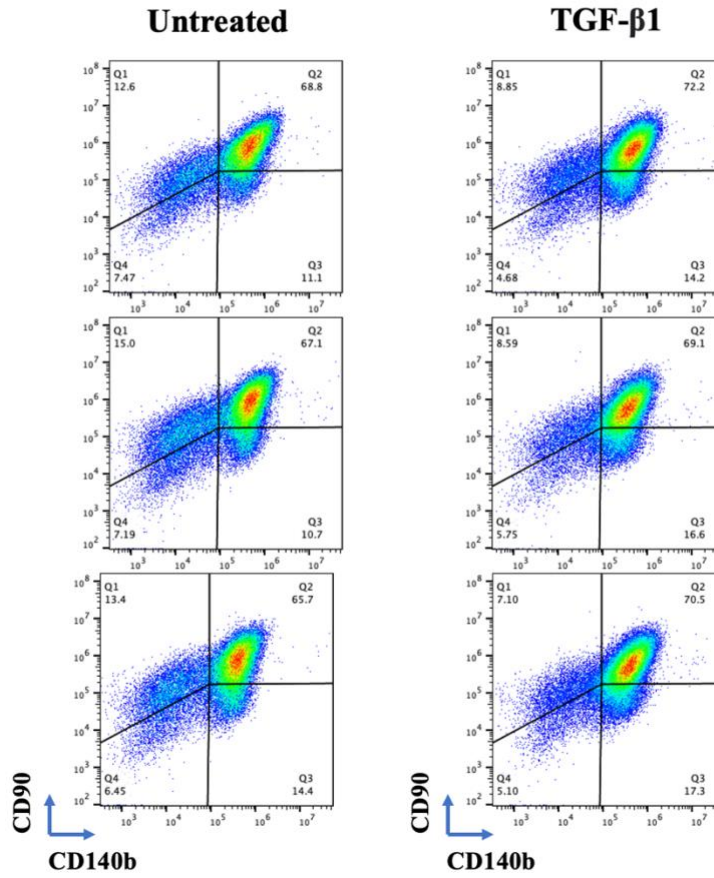


Figure 25: Cell density plots for TGF- β ⁻ (left) and TGF- β ⁺ (right) SMC samples. For all plots, going clockwise: Q1 contains CD90⁺CD140b⁻ cells, Q2 contains CD90⁺CD140b⁺ cells, Q3 contains CD90⁻CD140b⁺ cells, and Q4 contains CD90⁻CD140b⁻ cells.

FBs

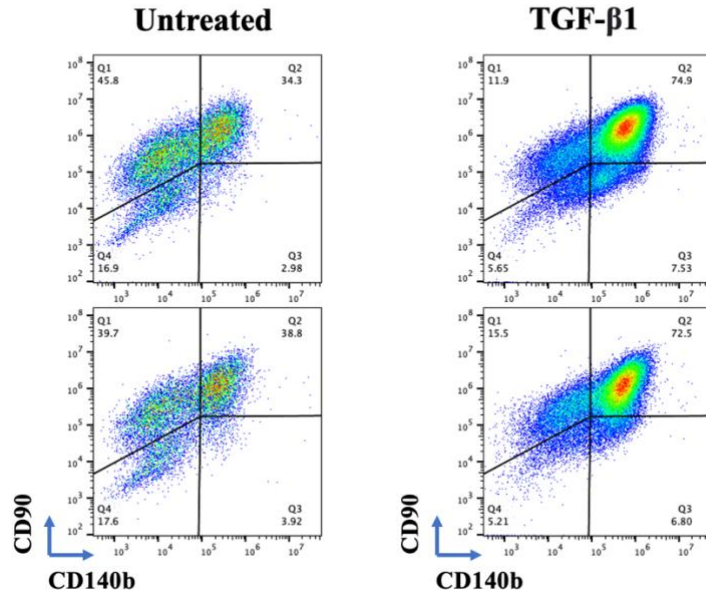


Figure 26: Cell density plots for TGF- β ⁻ (left) and TGF- β ⁺ (right) FB samples. For all plots, going clockwise: Q1 contains CD90⁺CD140b⁻ cells, Q2 contains CD90⁺CD140b⁺ cells, Q3 contains CD90⁻CD140b⁺ cells, and Q4 contains CD90⁻CD140b⁻ cells.

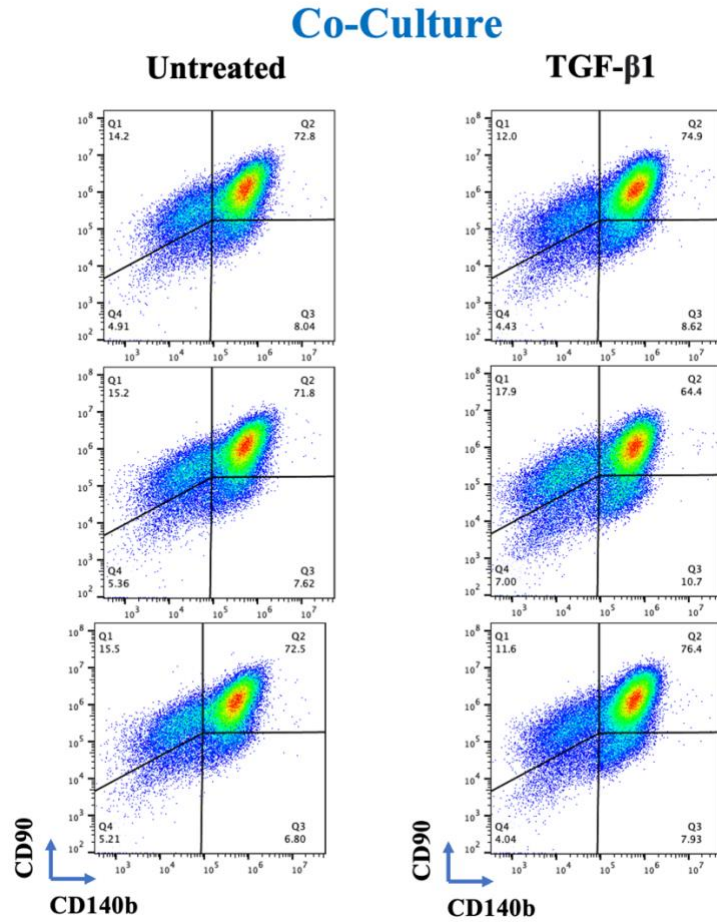


Figure 27: Cell density plots for TGF- β - (left) and TGF- β + (right) SMC & FB co-culture samples. For all plots, going clockwise: Q1 contains CD90⁺CD140b⁻ cells, Q2 contains CD90⁺CD140b⁺ cells, Q3 contains CD90⁻CD140b⁺ cells, and Q4 contains CD90⁻CD140b⁻ cells.

7.3. Engineering Standards

7.3.1. ASTM F3504-21

Standard Practice for Quantifying Cell Proliferation in 3D Scaffolds by a Nondestructive Method

This engineering standard discusses methods to measure cell viability and proliferation using methods that are not harmful to the cell samples. While the standard focuses on resazurin as the nondestructive reagent used to measure cell viability and proliferation, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was used

for this assay. Since cell viability was the specification of interest (instead of cell proliferation over time), cell viability was measured only at 0 and 4 hours. A 96-well plate was used to generate the calibration curve [38].

7.3.2. ASTM F3510-21

Standard Guide for Characterizing Fiber-Based Constructs for Tissue-Engineered Medical Products

As the Chaikof Lab looks towards using tri-layered tissue-engineered blood vessels (TEBVs) for patient engraftment, it will be important to characterize the graft using established characterization standards more thoroughly. This would also be important in the context of characterizing fibrosis [39].

7.3.3. ASTM F2603-06

Standard Guide for Interpreting Images of Polymeric Tissue Scaffolds

As imaging methods are optimized for tissue-engineered blood vessels (TEBVs), it will be important to follow engineering standards to interpret these images. This engineering standard was made for tissue scaffolds specifically made from collagen, such as the TEBVs fabricated in the Chaikof Lab [40].

7.4. Budget

Table 11: Updated Itemized Budget.

Item	Unit Cost	Amount Required	Total	Funding Source
Human Aortic Smooth Muscle Cells	\$934.00	1x1 vial	\$934.00	Chaikof Lab
Human Aortic Adventitial Fibroblasts	\$682.00	2x1 vial	\$1364.00	Chaikof Lab/ ES 100 Budget
Fungin™ Antifungal Reagent	\$161.00	5x1.5 mL vial	\$161.00	Chaikof Lab
Alexa Fluor™ 488 Phalloidin	\$588.00	1x300 units	\$588.00	Chaikof Lab
Stromal Cell Growth Media	\$177.00	4x500 mL bottle	\$708.00	Chaikof Lab
Rat Tail (for Collagen)	\$225.00	1x50 pack	\$225.00	Chaikof Lab
Hoechst Stain	\$115.00	1x5 mL vial	\$115.00	Chaikof Lab
Calcein & ethidium homodimer-1(Live/dead) Kit	\$576.00	1x1 kit	\$576.00	Chaikof Lab
MTS Assay Kit	\$185.00	1x250 tests	\$185.00	Chaikof Lab
Transforming growth factor-beta	\$211.00	1x10 µg	\$211.00	Chaikof Lab
Maxima H Minus cDNA Synthesis Master Mix	\$364.00	1x50 rxns	\$364.00	Chaikof Lab

TaqMan Fast Advanced Master Mix	\$538.00	1x5 mL	\$538.00	Chaikof Lab
TaqMan Probes: RPLP0, ACTA2, MYH11, & COL1A1s	\$215.00	4x250 rxns	\$860.00	Chaikof Lab
CellTracker™ Red CMTPIX Dye	\$493.00	20x50 µg	\$493.00	Chaikof Lab
CellTracker™ Deep Red Dye	\$493.00	20x15 µg	\$493.00	Chaikof Lab
PE CD 90 Antibody	\$325.00	1x100 tests	\$325.00	Chaikof Lab
APC CD140b Antibody	\$429.00	1x100 tests	\$429.00	Chaikof Lab
Genipin	\$131.00	1x25 mg	\$131.00	Chaikof Lab
Collagenase IV	\$331.00	1x1 g	\$331.00	Chaikof Lab
DNase I	\$62.90	1x10 mg	\$62.90	Chaikof Lab
TOTAL	\$7,235.90		\$9,093.90	