Role of Local Renin-Angiotensin System in Potentiating Early Calcific Aortic Valve Disease

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Role of Local Renin-Angiotensin System in Potentiating Early Calcific Aortic Valve Disease

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Biomedical Engineering

by

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University of Arkansas
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This thesis is approved for the recommendation to the Graduate Council.

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ABSTRACT

Angiotensin-II (Ang-II), a peptide hormone, is a potent vasoconstrictor and cell mitogen. It has been implicated in the development of hypertension as well as atherosclerosis. Recent work has shown that sclerotic aortic valves possess expression of angiotensin type I receptor (AT-1R) and angiotensin converting enzyme (ACE), suggesting altered angiotensin signaling during disease. The role of altered angiotensin signaling on aortic valve mechanics, however, is not clearly understood. We seek to understand the direct effects of the renin angiotensin signaling (RAS) system on the biological and biomechanical properties of aortic valve tissue and develop a finite constitutive model that mimics the effects of RAS on aortic valves. Our results showed that the mechanical properties such as stiffness were altered by RAS mediators. Three phenomenological constitutive models were utilize to characterize the biomechanical changes that occur due to RAS mediators on aortic valves, and the Fung-type model was shown to be the best fit model for the experimental data. Tissue maintained in anisotropy behavior, but the cross-coupling of the fibers was affected. Immunohistochemistry (IHC) showed that RAS affects the phenotypic properties of the cells in the aortic valve tissues. Picrosirius red (PSR) staining suggests that RAS mediators affect the production of collagen fibers, and quantitative polarized light imaging (QPLI) demonstrated that RAS mediators affect the orientation of collagen fibers. We concluded that RAS mediators affected the biological and mechanical functions of the aortic valve leaflet. The activation of VICS and increased production and disorganization of collagen fibers correlated to the stiffness of the tissue. These are all hallmarks of early disease progression, and in the future, we will further investigate the effects of RAS mediators in mechanics of the valve leaflet at the cellular level.
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CHAPTER 1 INTRODUCTION

Cardiovascular disease (CVD) is one of the most prevalent diseases worldwide and accounts for almost one third of deaths globally (1). Heart valve disease is the most common cardiovascular disease on the left side of the heart due to the higher pressure the valves experience (2). Aortic stenosis (AS) is the second most prevalent adult valve disease in the United States, accounting for approximately 4% of patient 45 years or older (3). If left untreated, AS can become fatal. Calcific aortic valve disease (CAVD) is the most common form of stenotic aortic valve disease. CAVD accounts for the majority of severe aortic stenosis in cases in which the patient is 70 years or older (4). Valve leaflets become thicker, fibrosed, and calcified as the disease progresses making them stiffer and narrowing the orifice of the valve. The number of valve replacements has doubled in the United States in the past decade, and surgery is the only option as there is currently no effective medical treatments for CAVD (5). There is a need to understand the pathophysiology of AS, and research is needed to understand the mechanisms of valve disease in order to develop new treatments/therapeutic strategies for CAVD. Angiotensin converting enzyme (ACE), angiotensin II (ang-II), and type 1 ang-II receptor (AT-1R) are present in stenotic aortic valves, strongly implying that the renin angiotensin system (RAS) signaling pathway is a factor in the disease progression (6). The fibrotic process is thought to be modified by RAS. Ang-II increases the amount of collagen present in the leaflet and in turn affects its mechanical properties. Ang-II has also shown to increase the stiffness of aortic valve leaflets and decreases their extensibility (7).

Biaxial testing has been shown to be the ideal method to mechanically-characterize soft incompressible materials such as biological tissues. Biaxial testing is where a material is subjected to load in both the x and y direction in contrast to uniaxial testing which only subjects
the material to load in one direction. In biaxial testing, Green strain (E) is calculated taking into account the deformation the tissue experiences in contrast to the engineering strain which only calculates the change in length the tissue undergoes. There are three stresses that take into account the orientations, force and area, that arise due to the tensorial nature of stress: Cauchy stress, 1st Piola-Kirchoff (P-K) stress, and 2nd P-K stress. Stress and strain information can be used to develop phenomenological constitutive models. These constitutive models can provide additional information on the mechanical responses of material. There are various constitutive forms that exist for soft biological tissues, and these are explored in this thesis.

To evaluate the potential for targeting RAS as a therapeutic strategy against valve disease, the objective of this thesis is thus to investigate how the renin-angiotensin signaling pathway affects the acute biological and biomechanical function of the aortic valve by investigating the role of angiotensin receptors (AT-1R and AT-2R) to which ang-II binds to, and the enzymes (ACE and chymase) that convert ang-I to ang-II. The first aim focuses on quantifying the biomechanical effects of RAS via biaxial mechanical testing of porcine aortic valve tissues pretreated with mediators of RAS signaling. Tissue tangent modulus, extensibility, areal strain, and anisotropy index are quantified to assess if RAS inhibition altered tissue mechanical properties. In the second aim, three non-linear phenomenological constitutive models were used to characterize the mechanical behavior of aortic valve tissues pretreated with RAS mediators. The third aim focuses on investigating the acute biological effects that RAS inhibition had on aortic valve tissue. The phenotypic changes the cells undergo on the tissue due to RAS were investigated via immunohistochemistry. The changes in collagen fibers undergo were investigated with quantitative polarized light microscopy and picrosirius red staining.
CHAPTER 2 BACKGROUND AND SIGNIFICANCE

2.1 The Heart

2.1.1 Anatomy

The heart is an important organ that acts as a pump and provides blood to the body. In a human, it is located in the center of the chest between the lungs and behind the sternum (Figure 2-1), but in other animals, location can vary slightly. The apex is the conical tip at the bottom of the heart that lies on the diaphragm that points to the left side of the body (8). The heart is separated into two sides by the intraventricular septum. The right side of the heart pumps deoxygenated blood from the body to the lungs and the left side pumps oxygenated blood from the lungs to the rest of the body. The heart is comprised of four chambers: the right and left ventricles and the right and left atria. The atria collects the blood, similar to a reservoir, and the ventricles pump the blood either to the lungs or the rest of the body (9). There are two coronary arteries, the left and the right, that provide blood to the heart itself (Figure 2-1). The superior and inferior vena cava are venous blood vessels that bring deoxygenated blood from the body to the right atrium. The superior vena cava carries the deoxygenated blood from the upper half of the body, and the inferior vena cava carries deoxygenated blood from the lower half of the body. The pulmonary artery carries the deoxygenated blood from the right ventricle to the lung to become oxygenated. The pulmonary veins then carry the oxygenated blood from the lungs into the left atrium. The aorta then delivers the oxygenated blood from the left ventricle to the rest of the body (8).
The heart contains four valves (Figure 2-2) which open and close to allow blood to flow in one direction. There are the atrioventricular valves (AV-valves) located between the atria and ventricles: the tricuspid valve (right side) and the mitral valve (left side). Then there are two semilunar valves that will allow the blood to leave the heart: the pulmonary valve (right side) and the aortic valve (left side) (10).

2.1.2 Physiology

Deoxygenated blood from the body is received by the right atria. Blood from the lower body enters the right atrium through the inferior vena cava and blood from the upper body enters through the superior vena cava. The deoxygenated blood then flows from the right atrium into the right ventricle through the tricuspid valve. Once the right ventricle has been filled, the tricuspid valve closes and the pulmonary valve opens to allow the right ventricle to pump blood
through the pulmonary valve into the pulmonary artery to the lungs. The lung then oxygenates the blood. The oxygenated blood then flows from the lungs to the right atrium by the four pulmonary veins. Similar to the right side, the blood flows from the left atrium into the left ventricle. The blood flows through the mitral valve, also known as the tricuspid valve, into the ventricle, and closes once the left ventricle has been filled with oxygenated blood. The left ventricle, the strongest chamber, then pumps the blood into the aorta when the aortic valve opens. The aorta then disperses the oxygenated blood to the rest of the body (11). When the aortic valve closes, oxygenated blood also flows through the coronary arteries. The left coronary artery provides oxygenated blood to the posterior area of the heart, and the right coronary artery provides oxygenated blood to the anterior area of the heart (Figure 2-3) (12).

**Figure 2-3. The physiology of the heart (8) and the cardiovascular system and organ blood supply (11)**

The circulation of blood through the heart is due to the contraction and relaxation of the heart, also known as the cardiac cycle (Figure 2-4). The relaxation state is also known as the
diastolic state where the ventricles fill with blood, and the contraction state is also known as the systolic state in which the blood is pumped either to the lungs or rest of the body. When the cardiac cycle begins, both the atria and ventricles are relaxed. Blood fills into the atrium and then the AV valve opens and the atrium contracts slightly to allow the blood to flow from the atrium to the ventricle. Once the ventricle is filled, the AV valve closes and the atrium returns to its relaxed state. The ventricle then starts to contract and the aortic valve opens to allow the blood to be pumped into the aorta. The left ventricle experiences a high amount of pressure during this time. Once all the blood is ejected from the ventricle, the aortic valve closes and the left ventricle returns to its relaxed state. The atrium starts to fill once more with blood and the cycle starts again (11).

Figure 2-4. The cardiac cycle and Wiggers diagram (11)

2.2 The Aortic Valve

2.2.1 Anatomy

The aortic valve allows oxygenated blood to flow from the left ventricle to the aorta and in turn to the rest of the body. The valve opens and closes with each heartbeat. It consists of 3 cusps or leaflets: the right coronary leaflet, non-coronary leaflet, and left coronary leaflet. The
right and left leaflets correspond to the left and right coronary arteries which are arteries that specifically provide blood to the heart (13). In the normal condition, each leaflet is less than 1 mm thick, smooth, flexible, and a mobile structure (14). During the systolic state, the aortic valve opens from 3 to 5 cm² (15).

The aortic valve predominately contains two cell types: valve endothelial cells (VECs) and valve interstitial cells (VICs). The VECs regulate valve anti-thrombogenicity, permeability, inflammatory cell adhesion, and paracrine signaling. The VICs maintain valve homeostasis and provide the strength and elasticity of the valve by secreting extracellular matrix (ECM) (Figure 2-5) (16). VECs line the surface of the leaflet and can undergo endothelial-mesenchymal transformation (EndoMT). On the inflow surface, they are oriented circumferential to the leaflet and have an anisotropic alignment. On the outflow surface, they exhibit no preferred orientation and have an isotropic alignment. This is due to the fact that they typically orient perpendicular to the flow, and in the outflow surface, the blood flow has an oscillatory blood flow (17). VICs are fibroblast-like cells but are phenotypically different from dermal fibroblast and have unique characteristics. Hence they are considered myofibroblast as they have characteristics of both fibroblast and smooth muscle-like cells (18). They adapt to their microenvironment, modulating their phenotype as a function mechanical force and soluble factors. VICs synthesize ECM components such as collagen, elastin, proteoglycans, and glycoproteins (19).

To meet the demands of the mechanical loading during each cardiac cycle, the valve is comprised of three different ECM fibrous layers with distinct matrices and mechanical properties: the fibrosa, spongiosa, and ventricularis (Figure 2-5) (5). The fibrosa is the layer closest to the aorta, contains type I and III collagen, and serves a load-bearing function. The spongiosa is the middle layer, contains glycosaminoglycan, and lubricates the other layers as
they shear and deform during each cardiac cycle. The ventricularis is the layer closest to the left ventricle, contains elastin fibers, and function to assure effective coaptation during diastolic closure (16). The leaflets are attached to the aortic root at the annulus which is a dense collagenous network. The annulus also dissipates the mechanical force the valve leaflets experience (5).

Figure 2-5. The architecture of the aortic valve (16)

2.2.2 Mechanical forces experienced by the aortic valve

The aortic valve experiences normal, shear and bending stresses. Normal stress is the stress perpendicular to the surface, and shear stress is the stress parallel to the valve surface. During systole, the ventricular side of the aortic valve is exposed to laminar shear stress (Figure 2-6a). During the diastole, the aortic side of the aortic valve mostly experiences oscillatory shear stress (Figure 2-6b). The left and right coronary cusps partially experience laminar shear stress while the non-coronary cusp only experiences oscillatory shear stress during diastole (Figure 2-6c) (13). The shear stress is highest on the left and right coronary arteries in comparison to the non-coronary, because they experience the blood flow to the coronary arteries (5). The circumferential and radial length is also longer during diastolic state in comparison to the systolic state. The strain the valve experiences during the cardiac cycle is due to the axial blood pressure, the bending forces, and the changes in tissue length (Figure 2-6) (13).
The fibrosa and ventricularis layers of the leaflet contain collagen and thus these layers bear the load on the valve during each cycle. The ventricularis contributes to the low strain/stress behavior, and the fibrosa contributes to the high strain/stress behavior. The mechanical response of the valve also differs in the circumferential (fiber orientation) and radial (cross-fiber orientation) direction. The radial direction undergoes higher strain than in the circumferential direction (20). Stella et.al showed that the fibrosa layer alone showed similar mechanical responses as an intact leaflet in the circumferential direction and only slightly less extensibility in the radial direction. The ventricularis layer however showed different mechanical responses to the intact leaflet. It had a longer toe region in both the circumferential and radial direction implying a compliant equibiaxial behavior (21).

2.3 Cardiovascular Disease

Cardiovascular disease (CVD) is one of the most prevalent diseases in the United States and the number one cause of death (1). CVD is any abnormal condition characterized by dysfunction of the heart and/or blood vessels. The severity of heart disease can vary from minor/unnoticeable to life threatening. There are two types of heart disease: congenital and acquired. Congenital heart disease is a genetic condition that comes from birth. There are certain
congenital diseases that can be unnoticed by the person their whole life and there are others so severe that if not treated immediately could cause death days or even hours after birth (22).

Acquired heart disease is developed with time during a person’s lifetime. Some risk factors for acquired CVD include: smoking, alcohol, physical inactivity, diabetes, overweight/obesity, high blood pressure, high cholesterol, and old age (23).

2.3.1 Heart valve disease

Heart valve disease is most common on the left side of the heart due to the higher pressures the left-sided valves experience. There are two types of valve diseases: valvular stenosis and valvular insufficiency/regurgitation (2). Both types may either be an acquired disease or a congenital disease. Valvular stenosis is when the leaflets thicken and in turn cannot open properly. Valvular regurgitation is when the valve cannot close properly thus causing back flow (2). This is most common in the mitral valve. However, the combination of aortic stenosis and regurgitation is much higher than each individually (2).

Aortic stenosis (AS) is the second most prevalent adult valve disease in the United States, accounting for approximately 4% of patient 45 years or older (3). If left untreated, AS can become fatal with an average survival of 2 to 3 years and an annual mortality of 25% (24). As AS manifests and the leaflet thickens, the aortic valve area (AVA) begins to decrease to approximately 2 cm² in contrast to a healthy valve’s AVA of 3 to 5 cm². The average decline in the AVA as AS progresses is 0.1cm² and an increase of 5 to 8mmHg in the mean gradient across the valve (15). The narrowing of the aortic valve prevents blood to flow from the left ventricle to the aorta properly. In elderly patients, aortic stenosis can be due to atherosclerosis and calcification (25). In contrast to a healthy valve in which leaflets are smooth, flexible, and mobile, the leaflets in valves that experience aortic stenosis become thickened, fibrosed, and
calcified. This reduces the mobility in the leaflet and can lead to valvular obstruction (14). It also increases the valve stiffness and narrows the valve orifice (5).

AS is similar to atherosclerosis which commonly affects the elderly (26). AS is very similar to the early stages in atherosclerosis in which the valve experiences an increase in mechanical stress and altered fluid shear stress due to endothelial damage (5). At the cellular level, VICs transition to osteoblast-like bone forming cells due to their activation, VECs undergo endothelial-to-mesenchymal transformation (EndoMT) due to their dysfunction or denudation, and the ECM forms calcific nodules. Inflammatory and immune cells infiltrate the valve and inhibitors of calcification are downregulated (16).

The mechanisms for AS are still largely unknown, but the importance of determining the pathological changes is important for the development of therapeutic treatments that might block or reverse the disease process (27). As of now, there are no drug-based therapies. Current treatments include balloon valvuloplasty, surgical aortic valve replacement, and transcatheter aortic valve replacement (24). Balloon valvuloplasty is used in patients who cannot undergo surgical or transcatheter aortic valve replacement due to the high surgical risks. Long-term survival however is not changed. There is an approximate 50% survival at 1 year, 35% at 2 years, and 20% at 3 years. Each year in the United States, approximately 67,500 surgical aortic valve replacements are performed (24). They are performed with either mechanical or bioprosthetic valves. Though bioprosthetic valves do not require anticoagulants like mechanical valves, a study showed that there was no significant difference in 15-year survival or stroke in patients between the ages of 50-69 years old (24). In inoperable and high-risk patients, transcatheter aortic valve replacement has shown to be beneficial and reduce the mortality by 21.8%. There are several approaches including transfemoral, transapical, transaxillary,
transcarotid, transcaval, and transaortic. In high risk but operable patients, the 5-year mortality was similar in patients who underwent transcatheter or surgical aortic valve replacements (24).

2.3.2 Calcific aortic valve disease

Calcific aortic valve disease (CAVD) is the most common form of stenotic aortic valve disease. CAVD accounts for the majority of severe aortic stenosis in cases in which the patient is 70 years or older (4). In cases involving younger patients, aortic stenosis is due to an abnormal valve such as a bicuspid valve (4). The degree of the calcification is determined by the severity of the valve lesions, the progression of the disease, and the development of symptoms and adverse events (5). Risk factors of CAVD include hypertension, smoking, diabetes, elevated cholesterol, and male gender. The biological processes involved in CAVD include endothelial dysfunction and inflammatory responses which lead to the remodeling of the valves (28). As alluded to earlier, the only treatment for CAVD is aortic valve replacement. Medical therapies to prevent or decrease the progression of CAVD have not been possible as there is still a need to better understand the mechanical and biological process that cause CAVD, and to identify interventions to prevent the initiation and progression of CAVD (6).

The aortic valve of patients with CAVD become progressively thickened, fibrosed, and calcified. Throughout history, this has been thought to be a result of “wear and tear” and age-associated valvular degeneration. However, recent studies have shown that due to biochemical, humoral, and genetic factors, the CAVD occurs via an active disease process that includes inflammation which then leads to fibrosis and calcification. In areas of inflammation in the valve, endothelial injury or distribution has been observed. Lipoproteins are able to stimulate intense inflammatory activation, and are present in early aortic valve lesions. An increase in mechanical stress and reduced shear stress is believed to be due to endothelial damage. A
combination of these two events triggers inflammation in the valve. Inflammatory cells penetrate the valve and may then stimulate the valve to induce a fibrotic and calcific process (Figure 2-7). The inflammatory activity activates the fibroblast-like cells in the valve, and they differentiate into a more myofibroblast-like cell. This activity then increases the synthesis and degradation of fibrous tissue. Fibrous tissue and the remodeling of the ECM thickens the valve. The valve progressively stiffens affecting the mechanics of the valve. The disease then progresses as the myofibroblasts differentiate into osteoblast-like cells making the valve stenotic. Osteoblasts drive the calcification process. Calcific nodules form on the valve and contain a bonelike matrix of collagen, osteopontin, and other bone matrix proteins. Calcification is known to narrow the opening of the valve. CAVD develops which can be fatal to patients (5).

Figure 2-7. The process of the progression of healthy valves to CAVD diseased valves (5)

The trilayer structure of the valve is important in both the biological and mechanical function of the valve. As CAVD develops, the trilayer structure becomes disturbed and consequently its function is impaired. Type I collagen has features that regulate cellular behaviors. The length, thickness, alignment, and density of the collagen fibers can modulate cells proliferation and differentiation, and affect their cell polarity and motility (29). CAVD has been associated with the remodeling of the collagen fibers, which in turn affects the biological and
mechanical factors of the valve. In the fibrosa layer, fibers in a diseased valve are shorter than that of those in a healthy valve (30). As far as fiber number, width, density, and alignment, the diseased valve seems to have similar properties of that in a healthy valve (30). However, in the diseased spongiosa layer, the collagen fibers appear to become wider and denser thought with unchanged length (30). It can be speculated that the crosslinking of the fibers is affected in the fibrosa layer while the collagen production is affected in the spongiosa layer (30). In the spongiosa layer, there are more immature fibers while in the fibrosa layer there are more mature fibers (30). The increased production of collagen and the disorganization of the fibers are an indication of CAVD, and will be explored in this thesis.

2.3.3 Mechanical alterations in diseased valves

The velocity of blood flow through a diseased aortic valve is higher as the pressure gradient is higher (mean pressure gradient >40mmHg) between the aorta and left ventricle during cardiac systole due to the narrowing of the valve. A healthy valve experiences a peak velocity of 1.35 ± 0.35 m/s while the velocity in a valve that has developed AS can exceed 4 m/s (13). The increase in pressure, increase in forces, and changes in length of the valve increases the stiffness and hence decreases the strain. The stiffness reduces the opening of the valve which increases the axial pressure on the ventricular said and in turn changes the direction of the blood flow during the systolic state (13). The systolic pressure may then exceed the diastolic pressure, and the pressure gradient can be four to six times higher than normal. During calcification, the cusp loses its stretch and can also result in regurgitation, that is, blood flowing back from the aorta into the left ventricle (13). Echocardiography is a useful tool in diagnosing AS by providing clinical information such as peak velocity of blood flow through the valve, mean pressure gradient, and aortic valve area. It is essential to know the aortic valve area to determine AS because velocity
and pressure gradients are only flow-dependent variables. Changes in velocity and pressure gradients can also be attributed to other cardiovascular diseases such as impaired left ventricular contractility. In patients with severe AS, the aortic valve area is estimated to be \( \leq 1 \text{ cm}^2 \) (4).

The mechanical functions of the valve are affected by the collagen alterations in the trilayer structure (30). In a healthy valve, the collagen fibers in the fibrosa and spongiosa/ventricularis layers are oppositely aligned (30). In a diseased valve, the alterations to the fiber alignments seen in the fibrosa and the increased production of fibers in the spongiosa affect the mechanical load being applied by the mechanical stress the valve experiences. The stress that the fibrosa and ventricularis layers cause can apply friction between the layers, and when the density of the fibers increases in the spongiosa, the role of the spongiosa to minimize that friction is affected (30).

The VECs and VICs are able to actively respond to the biomechanical factors the valves experience. VECs are the first to experience the shear stress as they line the valve. The changes in shear stress alter VEC gene expression patterns (31). Unlike laminar shear stress, oscillatory shear stress does not induce the protective genes suggesting that the change in shear stress could be responsible for the changes in the VECs function (32). The different biomechanical forces the VECs experience in the aortic and ventricular side also contribute to the differential gene expression of the VECs located on either side of the valve. In the aortic side, the VECs have higher expression of genes associated with calcification, and in the ventricular side, VECs have higher expression of genes associated with inflammation (33). VICs are protected from shear stress by the endothelial layer, but they experience the changes in the stress and strain during the cardiac cycle. In \textit{in vitro} studies were cells were grown in statistic state, cells became hypersynthetic, and when exposed to flow, synthesis is maintained at normal levels suggesting
the importance of strain on VICs (34). The cell physiology is regulated by the mechanical forces and can affect the biosynthetic activity of the cells. Cyclic stretch can upregulate the expression of smooth-muscle markers such as α-SMA suggesting that VICs are becoming more myofibroblast-like or smooth muscle cell-like (35). Increase in VIC stiffness correlates with the biosynthesis of collagen proteins (18). The stiffness of the environment in conjunction with the differentiation of VICs can cause calcification (36). The cellular response to environmental mechanical forces needs to be further studied to have a deeper understanding of valve mechanobiology for the potential of therapeutic treatments (37).

2.3.4 Mechanisms of CAVD

The development and progression of CAVD is determined by active biological processes (38). VICs undergo cell differentiation and display osteoblast-like phenotypes. There are various signaling pathways including the Runx-2/NOTCH-1 signaling, Wnt-2/Lrp5-β catenin signaling pathway, and the renin angiotensin signaling (RAS) pathways that are thought to modulate the CAVD process (5). During disease progression, VICs become more bone-like and express osteoblast markers such as osteopontin, osteocalcin, bone morphogenetic proteins 2 (BMP-2) and 4 (BMP-4), and osteoblast-specific transcription factors (19). TGF-β1, a cytokine, is present in the extracellular matrix (ECM) in calcified aortic cusps. The accumulation of TGF-β1 could be due to the endothelial injury and inflammatory cell infiltration that VICs undergo which initiates apoptosis of the cells leading to calcification (39). In the presence of cyclic strain, the upregulation of TGF-β can alter ECM architecture and compromise the valve function (40). When strain is increased above the normal physiological state, VICs respond by increasing pro-inflammatory proteins such as VCAM-1, ICAM-1, and E-selection (41). Elevated cyclic pressure also upregulates the expression of VCAM-1 and down regulates the expression of OPN (42). The
renin-angiotensin system (RAS) plays a key role in promoting and maintaining inflammation, and it has shown to upregulate the expression in pro-inflammatory cell adhesion molecules such as VCAM-1 and ICAM-1 in vascular disease (43). Thus in this thesis, we seek to explore the how RAS affects the biomechanical functions of valve leaflets.

2.4 Renin-Angiotensin System

2.4.1 Renin-angiotensin signaling pathway

The renin-angiotensin system (RAS) plays a role in the regulation of blood pressure and is a regulatory system for renal and cardiovascular function (44-46). The RAS is a very complex signaling pathway (Figure 2-8).

Figure 2-8. The multiple pathways in the renin-angiotensin signaling system (47)

The central pathway in the activation of RAS occurs when angiotensinogen is cleaved by renin, a proteinase enzyme released by the kidney, to form angiotensin I (1-10) (ang-I). Ang-I is then converted rapidly to angiotensin II (1-8) (ang-II) by the angiotensin-converting enzyme
(ACE) or ACE independent pathways such as chymase (47). The concentration of angiotensinogen, ang-I, and ang-II was measured in the plasma and kidney of rat models. This study showed that the plasma concentration of angiotensinogen was much greater than the concentration of ang-I and ang-II implying that the active concentration of ang-II is a small fraction of what could be produced via the conversion of angiotensinogen to ang-I and ultimately ang-II (48).

Ang-II then binds to two different receptor subtypes, type 1 ang-II receptor (AT-1R) or type 2 ang-II receptor (AT-2R), to induce the downstream signaling effects (49). AT-1R is present in the kidney, heart, brain, adrenal cortex and vessel walls. It mediates the effects of ang-II in most cardiovascular function. AT-2R is mostly expressed in regions of the brain and adrenal medulla. Little is known over the role of AT-2R in cardiovascular function, however it is expressed in the vascular wall, cardiac ventricles, and valve (50, 51). AT-1R causes vasoconstriction, cell-proliferation, endothelial dysfunction, and hypertrophy whereas AT-2R causes vasodilation, anti-proliferation, and apoptosis which counterbalances the effects of AT-1R (Figure 2-9) (52-54).

**Figure 2-9. The local renin-angiotensin signaling pathway**

There are also non-angiotensin converting enzymes such as chymase and cathepsins also play a role in the conversion of ang-I to ang-II. The ability for chymase to generate ang-II in the heart tissue is not well known, however it is present in diseased tissue (55). RAS regulates intravascular volume, blood pressure, and tissue repair, and though can be protective, when
highly stimulated it can cause vasoconstriction, vascular smooth muscle proliferation, endothelial dysfunction, inflammation, fibrosis, and thrombosis (50, 56).

2.4.2 Renin angiotensin system inhibitors

Extensive research has been conducted on inhibiting RAS to treat hypertension, renal failure, cardiac failure, atherosclerosis, and myocardial infarction such as renin inhibitors, ACE inhibitors (ACEIs), ang-II receptor blocking agents (ARBs), and chymase inhibitors. By blocking RAS, the formation or binding of ang-II can be prevented and in turn reducing its downstream effects (57, 58).

ACEIs were discovered in the late 1970s and have been an effective treatment in various cardiovascular diseases such as hypertension and left ventricular dysfunction. ACEIs prevent ang-I from converting into ang-II (59, 60). They have been shown to improve endothelial function and have anti-atherosclerotic, anti-inflammatory, anti-proliferative, and antioxidant properties (61, 62). Not all ACEIs can be used to treat both hypertension and heart failure but drugs such as lisinopril, captopril, and quinapril have shown to help greatly with both (44). However, by targeting ACE there can be an upregulation of ang-I, and ang-I could still be converted into ang-II through an ACE independent pathway such as chymase (55). Chymase inhibitors such as chymostatin alone or in combination with ACEIs have been used to treat atherosclerotic human coronary arteries but further research needs to be conducted on other effects of ang-II on cardiovascular disease (63).

Due to the ability for ang-I to convert to ang-II even with ACEIs, ARBs are also used to treat RAS-induced disease. ARBs block ang-II from binding to angiotensin receptors such as AT-1R. Losartan is an ARB that inhibits ang-II from binding to AT-1R. By blocking AT-1R, the harmful effects of ang-II such as vasoconstriction and cell proliferation are eliminated (55). An
ELITE study of over 3,000 patients compared losartan to captopril by randomizing the patients and assigning them to either treatment (64). Mortality, cardiac death, and hospital admission did not differ significantly between both treatments, however, there were many more patients that withdrew from the captopril treatment due to adverse side effects. This study showed that losartan was a more tolerable treatment that captopril, and that ARBs can be an effective treatment for those patients that are not able to be treated with ACEIs (64).

2.4.3 Renin angiotensin system and valve disease

Studies have shown that ACE is not present in normal aortic valves but is present in stenotic valves (65). ACE is carried by low-density lipoprotein (LDL) particles and enters the lesions in the stenotic valve from circulation bound to it (66). In addition to ACE, ang-II and AT-1R are also present in stenotic aortic valves (28). Though ang-II is present and produced in other parts of the body, the presence of ACE in stenotic valves suggests that ang-II is being converted locally. This observation implied that the RAS signaling pathway is a factor in the valve disease process (6). The fibrotic process is thought to be modified by RAS. Stenotic valves have an upregulation of ACE and ang-II, and AT-1R is present on valve myofibroblasts. The binding of ang-II to AT-1R can lead to an increase in inflammatory responses, inducing the cells within the valve to differentiate into osteogenic-like cells (5).
Myles et al. demonstrated that ang-II increased the elastic modulus in the radial direction of the aortic valve via biaxial mechanical testing. They also showed that ang-II decreased the extensibility of the leaflets which correlates with an increase in stiffness. In addition, ang-II increased the amount of collagen present in the leaflet. It can be implied that the increase in collagen synthesis affects the mechanical properties of the tissue as it correlates with the increased stiffness and inversely correlates with decreased extensibility (7).

Even though ACE inhibitors and angiotensin receptor blockers are well known to reduce hypertension, the impact they may have in the context AS is not well studied (5). It has been suggested as a potential therapeutic for CAVD, and studies are currently trying to find the correlation between the inhibitors and AS (27, 67).
To evaluate the potential for targeting RAS as a therapeutic strategy against valve disease, the objective of this thesis is thus to investigate how the renin-angiotensin signaling pathway affects the biological and biomechanical function of the aortic valve by investigating the receptors (AT-1R and AT-2R) in which ang-II binds to and the enzymes (ACE and chymase) that convert ang-I to ang-II.
CHAPTER 3 MOTIVATION AND SPECIFIC AIMS

3.1 Motivation of Study

In a previous study conducted by us, the interaction between serotonin (5HT) and angiotensin II (ang-II) were investigated to understand their effects on aortic valve mechanics and remodeling (68). In this previous study, there were four treatment groups: control, 5HT, ang-II, and 5HT/ang-II. VICs were assayed for cellular contractility, cytoskeletal organization, and collagen remodeling, and in an in vivo mouse model, blood pressure was monitored and echocardiography were performed in addition to histology and immunohistochemistry. Results suggested that the interaction of 5HT and ang-II altered the function and remodeling of the valve, but our results also showed that ang-II alone had detrimental effects on the aortic valve. In vitro, VICs treated with ang-II had a higher actin orientation parameter and COL1A1 mRNA expression. In-vivo, ang-II treated mice had a higher blood pressure, ejection fraction, end-diastolic volume, and end-systolic volume after 3 weeks of treatment. Picrosirius red staining showed that ang-II had a higher percentage of thick fibers in the leaflet attachment region to the aortic root indicating collagen remodeling (Figure 3-1).

Figure 3-1. (A) H&E staining and (B) Picrosirius red (PSR) staining (scale bar = 200µm). Arrows point to the valve free edge. Valve attachment zone labeled “a”. (C) Quantification of PSR stain for valve attachment zone. (* p<0.05) (68)
Immunohistochemistry showed an increase of expression of Ki-67, α-SMA, TGFβ1, collagen III, and AT-1R indicating ECM remodeling (68). This prior work is the primary motivation for us to further investigation the renin angiotensin system (RAS) signaling pathways, and its effects in both the cellular and tissue level on the aortic valve (Figure 3-2).

![Figure 3-2. Representative micrographs of samples immunohistochemically labeled with Ki-67, α-SMA, TGFβ1, AT-1 receptor, and collagen type III. Arrows indicate the aortic valve leaflets. The aortic root is indicated by the letter “a”. (scale bar=100µm) (68)](image)

There has been research conducted by others on the influence of ang-II on the biomechanical properties of aortic valves. Biaxial testing showed that ang-II increased the stiffness of aortic valve leaflets, and it was believed that an increase in collagen content altered valve mechanical properties (7). Studies have also shown that stenotic aortic valves have an
increase in expression of angiotensin converting enzyme (ACE) and type 1 angiotensin II receptor (AT-1R) in comparison to healthy valves (28). These results suggest that RAS may participate in the progression of aortic stenosis and that blocking RAS can serve as a therapeutic strategy (65). Towards this end, further studies need to be conducted to investigate the role of ang-I and ang-II signaling on the biomechanical properties of the aortic valve. Our collaborators in Imperial College have studied the role of RAS in cardiac fibroblasts, calcific aortic valves, blood vessels, radial arteries, and internal thoracic arteries, and have shown that RAS mediators can be inhibited using ACE inhibitors (ACEIs) quinaprilat, receptor antagonists losartan and PD123319, and the chymase inhibitor chymostatin (Figure 3-3) (63, 69-71).

![Diagram of RAS pathway]

**Figure 3-3. Inhibitors of the renin angiotensin system receptors and converting enzymes**

To evaluate the potential for targeting RAS as a therapeutic strategy against valve disease, the **objective** of this thesis is thus to investigate how the renin-angiotensin signaling pathway affects the biological and biomechanical function of the aortic valve by investigating the receptors (AT-1R and AT-2R) in which ang-II binds to and the enzymes (ACE and chymase) that convert ang-I to ang-II. It is hypothesized that a local renin-angiotensin signaling exists within the valve milieu and affects the biological and mechanical function of the aortic valve.

The **first aim** focuses on quantifying the biomechanical effects of RAS via biaxial mechanical testing of porcine aortic valve tissues pretreated with the aforementioned mediators.
of RAS signaling (Figure 22). Tissue tangent modulus, extensibility, areal strain, and anisotropy index are quantified to assess if RAS inhibition altered tissue mechanical properties.

In the **second aim**, three non-linear phenomenological constitutive models were used to characterize the mechanical behavior of aortic valve tissues pretreated with RAS mediators. The **third aim** focuses on investigating the biological effects that RAS inhibition had on aortic valve tissue. The phenotypic changes the cells undergo on the tissue due to RAS were investigated via immunohistochemistry. The changes in collagen fibers undergo were investigated with quantitative polarized light microscopy and picrosirius red staining.

The next three sections of this chapter will give a more detailed overview of each aim.

**3.2 Specific Aims 1:** Quantify the biomechanical effects of mediators of the renin angiotensin signaling (RAS) system on aortic valves.

In this aim, we sought to quantify the biomechanical effects of RAS on aortic valve tissues through biaxial mechanical testing. There are two commonly utilized methods for the tensile testing of soft biological tissues – uniaxial and biaxial mechanical testing. Uniaxial testing tests the sample in one direction, and is commonly used for failure testing of isotropic or transversely isotropic materials. However, biaxial mechanical testing is a more effective way of
testing anisotropic material, directionally dependent material, and for developing phenomenological constitutive models to characterize their material behavior.

We sought to understand the effects of RAS on valve tissues by testing the ability of ang-II to bind to the AT-1R and AT-2R sub-types, and the ability for ang-I to convert to ang-II via ACE and chymase activity. To do so, tissue was treated for 48 hours with either ang-I or ang-II alone to understand their direct effect. 24 hours prior to being treated with ang-I or ang-II valve leaflets were treated with only RAS mediator inhibitors, and tissue was the co-treated with ang-I or ang-II and the inhibitors for 48 hours. To understand the binding to the AT-1R and AT-2R receptor subtypes, tissue was treated with ang-I or ang-II in combination with AT-1R or AT-2R inhibitors, losartan and PD123,319 respectively. To understand the conversion of ang-I to ang-II, tissue was treated with ang-I in combination with the ACE and chymase inhibitors, quinaprilat and chymostatin, respectively. After appropriate treatment, the tissue was tested via biaxial tensile testing at five different tension ratio protocols at a maximum membrane tension of 90 N/m: $T_{\text{circ}}:T_{\text{rad}} = 1:1, 1:0.75, 1:0.5, 0.75:1, 0.5:1$.

The Green strain and the 2\textsuperscript{nd} Piola-Kirchhoff (2\textsuperscript{nd} P-K) stress were calculated for each individual sample. Mechanical characteristics of the individual samples and the average model were first calculated using the equibiaxial protocol. The stiffness of a sample was characterized with the low and high tangent modulus for both the circumferential (fiber direction) and radial (cross fiber direction) direction. From the gradient of the high tangent modulus, the extensibility of the tissue was calculated. The higher the extensibility, the more strain the sample underwent for the given maximum tension. With the maximum Green strain in the radial and circumferential direction, the areal strain and the strain anisotropy index was calculated. An
anisotropy index close to one indicated that the sample was more isotropic, whereas an
anisotropy index greater than or less that one indicated that the sample was more anisotropic.

3.3 Specific Aims 2: Utilize phenomenological constitutive models to characterize the
biomechanical changes that occur due to RAS mediators on aortic valves.

In this aim, three finite constitutive models were used to characterize changes in the
mechanical properties of the aortic valve samples due to RAS mediators: modified Mooney-
Rivlin (M-R) isotropic model, modified M-R anisotropic model, and Fung-type model. The
Fung-type constitutive model is typically used to characterize the multiaxial behavior of a soft
anisotropic material, while the Mooney-Rivlin constitutive model is usually used to characterize
isotropic materials. We fit our biaxial mechanical data to these phenomenological models to
better characterize the mechanical changes that occurred due to the RAS mediators.

3.4 Specific Aims 3: Investigate the biological effects of mediators of the RAS system on aortic
valves.

In this aim, we sought to understand the biological effects that mediators of the RAS
system had on aortic valve tissues. Tissue received similar treatment as in specific aim 1. The
changes in valve cell phenotype was analyzed through immunohistochemistry (IHC) by
qualitatively analyzing the expression of certain proteins known to be correlated with the
valvular interstitial cells (VICs) during valve disease progression. The alterations of collagen
fiber due to RAS mediators was quantitatively analyzed with quantitative polarized microscopy
(QPLI) and picrosirius red (PSR).
CHAPTER 4 SPECIFIC AIM 1

Quantify the biomechanical effects of mediators of the renin angiotensin signaling (RAS) system on aortic valves

4.1 Introduction

Aortic stenosis (AS) is one of the most common valve diseases in the western hemisphere (3). Mechanical stress is thought to be one of the factors central to its progression (13). The renin angiotensin signaling (RAS) system has been implicated in the progression of aortic valve disease as activated RAS can induce fibrosis within the valve (5), altering the mechanical stress that the valve experiences, accelerating the progression of the disease (14). In addition, the endothelial damage observed in the early stages of AS increases the mechanical stress and alters the fluid shear stress on the valve (5). Calcific aortic valve disease (CAVD) is induced by the long-lasting mechanical stress and is a degenerative and currently irreversible process (6). The mobility of the leaflet becomes restricted as the calcification progresses (3).

The aortic valve is comprised of three different layers: the fibrosa, spongiosa, and ventricularis. This structure is important in the biological and mechanical function the valve (30). The calcific nodules and lesions that occur due to CAVD are primarily in the fibrosa layer (16). The progression of this disease can compromise the integrity of the trilayer surface and consequently its biological and mechanical function. In a healthy valve, the collagen fibers in the fibrosa and spongiosa/ventricularis layers are oppositely aligned, and the alterations to the fiber alignments and production seen in the diseased fibrosa and spongiosa layer affect its mechanical function (30).

Planar biaxial mechanical testing is the ideal method to assess changes in mechanical properties for incompressible materials such as aortic valve tissue as it allows for the
quantification of a two-dimensional stress-strain relationship. Biaxial testing is where a material is subjected to load in both the x and y directions in contrast to uniaxial testing which only subjects the material to load in one direction (72). In anisotropic materials, uniaxial testing is not recommended. Mechanical responses in aortic valve leaflets are dependent on the direction of the load. In a leaflet, the collagen fibers are oriented in the circumferential direction, and thus has a larger stiffness in the circumferential direction in comparison to the radial direction (73). When stretched in the circumferential direction, the tension in the fibers prevent the collagen fibers from further stretching as the fibers are aligned. In the radial direction, the collagen fibers provide less resistance and hence have a lower stiffness in that direction (7). In biaxial testing, an equibiaxial loading and proportional loading protocols are used to for mechanical characterizations. Equibiaxial loading is where the same load is applied in both direction, and proportional loading is where the load is kept at a constant proportion between both directions (73). Using equibiaxial loading experimental data, mechanical properties of the leaflets can be calculated such as extensibility, tangent modulus, and degree of anisotropy. (74).

This aim focuses on quantifying the biomechanical effects of RAS signaling on the aortic valve via equibiaxial mechanical testing. Tissue tangent modulus, extensibility, areal strain, and anisotropy index will be calculated using equibiaxial experimental data from biaxial tests. The average 2\textsuperscript{nd} Piola-Kirchoff stress and Green strain data was used to determine changes in anisotropy due to due to RAS.
4.2 Materials and Methods

Protocols can be found in Appendix A. MATLAB codes and instructions can be found in Appendix B.

4.2.1 Tissue treatment

Fresh porcine hearts (3-6 months old) were provided by Cockrum’s Custom Meat Processing and Taxidermy (Rudy, AR) or Braunschweig Processing (Neosho, MO), both FDA-approved abattoirs. They were transported in cold sterile dPBS. The hearts were then dissected aseptically, and the left, right, and non-coronary aortic valve leaflets were pooled and washed in dPBS. They were then incubated with 150 U/ml penicillin/streptomycin in PBS for 10 mins.

The leaflets were then cultured in DMEM supplemented with 2% FBS, 150 U/ml P/S, and HEPES. The binding of ang-I and ang-II to the receptors was investigated by inhibiting AT-1R or AT-2R. The samples were treated with either AT-1R inhibitor losartan (1μM) or AT-2R inhibitor PD123-319 (1μM) for 24 hour before being treated in combination with ang-I (10 μM) or ang-II (10 μM) for an additional 48 hrs (69). The local conversion of ang-I to ang-II was investigated by inhibiting ACE or chymase. Samples were treated and tested as outlined before but with ACE inhibitor quinaprilat (1 μM) or chymase inhibitor chymostatin (10 μM) and only ang-I and not ang-II. Tissue for biaxial mechanical testing was immediately tested following the treatment period (69).

4.2.2 Biaxial mechanical testing

Samples were cut into 1cm² square specimens aligned in the radial-circumferential directions. A four-marker array was then placed in form of beads on the tissue to track the displacement of the tissue. Tissue was mounted onto a TestResources biaxial testing device (Figure 4-1) with zinc-plated steel wires with the radial direction aligned with the y-axis and
circumferential direction aligned with the x-axis. The specimen was then immersed into a saline bath at 37°C (Figure 4-2).

**Figure 4-1.** Biaxial mechanical testing device.

**Figure 4-2.** Sample mounted on biaxial testing device.
The specimen was brought to a tare load of 0.05 N along each axis. The system was operated in the position-based settings at a data acquisition (DAQ) rate of 100 Hz and cycle rate (frequency) of 1 Hz. Specimen was then preconditioned by cycling 20 times to a membrane tension of 90 N/m. Mechanical testing in the circumferential and radial axes consisted of the following tension ratios with a maximum tension of 90 N/m: \( T_{\text{circ}}:T_{\text{rad}} = 1:1, 1:0.75, 1:0.5, 0.75:1, 0.5:1 \). Tissue was then stretched three times, and data acquired. Only the 1:1 tension data was used in this aim, while the entire data set was used in aim 2 for constitutive modeling.

A camera placed above the sample captured images at 100 Hz while the tissue was being stretched. These images were later used to detect the displacement of the beads in the four marker array to calculate deformation.

### 4.2.3 Stress and strain calculations

The MATLAB code Biaxial_1 was used to determine which stretch cycle had the closest desired maximum load (0.9N) for analysis. The code works in the following way. Before running the code, make sure that columns 2-5 in the csv file output by the system correspond to force x1, y1, x2, and y2, and then delete all words in the file as it won’t be read correctly otherwise. The user then runs the code and is then asked to select the csv file. Note: If the system being used exports an excel file, the code needs to be changed to read an .xls files instead. Next the code plots the force in all axis and the average of the x and y axis. By doing this, the user can see how many stretches were completed during the test being analyzed. Usually, only three stretches are done. However, there were times that the system would falter and export fewer data sets. To overcome this, sometimes the sample would have to be stretched more than 3 times to get the data of at least the first 2 to 3 stretches. The user is then asked to input how many stretches were done. The code then finds the peaks of the x2, and then sorts them in a descending order to find...
the n number of stretches. It indexes the row in which they were found and sorts them in ascending order. This is done because the max force for each peak varies, and if you use the order in which they are found, the stretches would be in order of max force and not in order that they were done.

A cycle is done at a DAQ of 100 Hz and frequency of 1 Hz so 100 data points are acquired for each cycle. The first 50 data points are the loading curve and the last 50 data points are the unloading curve. The maximum force reached is point 50 in the cycle. To zero the force, force at point 50 is subtracted by force at point 1 in the cycle. To do so, the maximum force in the row indexed for the stretch is subtracted by the force indexed 49 data points before it. The force from each stretch of the x and y axis is displayed in the command window so the user can choose the stretch to analyze. Note: The x1 loading cell was not working properly so the force showed is not an average of x1 and x2 but just x2.

Prior to running MATLAB code Biaxial_2, the images acquired from the camera were separated into individual folders from each stretch. This is done by going through the images and separating the 100 images that correspond to each stretch. Before running the code, the user must change lines 51-53. Line 51 is the path to the folder containing the images (path1=), line 52 is the file name of the first image excluding the last 3 numbers (name1=), and line 53 is the pathname for the folder containing the MATLAB code in which the information will be outputted to (out1). Add “\point” to the end of the path name in line 53 (out1). Lines 51 and 52 will change for every run, but line 53 will stay the same if the MATLAB code run is in the same folder/path.

The user can then run the program. The user is first asked to input the number of the first image in the command window. 100 images will be analyzed so to calculate the number of the
last image, so 99 is added to the number of the first image to index the numbers of the 100 images being analyzed. The first image is then opened. The code is written to track 4 points so the user clicks the 4 points that will be tracked. The user must choose the area in the center and must be chosen in the following order:

```
3--------4
/    /    \\
/    /    \\
2--------1
```

After clicking the 4th point, the code opens the second file and used the function automarkertrack to track the distance the points have moved to from the previous image by analyzing a set distance of pixels from the point chosen. The optimal pixel number was 50. The code then writes the x and y position of every point chosen into a text file (point1.txt, point2.txt, point3.txt, and point4.txt) which is exported in the MATLAB folder. The code then reads the files and makes a 100x8 array containing the x and y positions of each point for all 100 images. The distance the point has moved is calculated by subtracting the initial coordinates of the points in the first image. An isoperimetric equation is used to compute the strain at the center of the element and the element of the Jacobian. The elements of the deformation gradient (F) were computed using the Jacobian factor and isoperimetric strain-differentials. Green strain (E) is calculated using F with the following equation: \( E = 0.5(F^TF - 1) \). It then creates an excel file (deformresults.xlsx) containing the Exx, Eyy, Exy, Ezz, E1, E2, dir1, and dir2. It also saves the deformation gradient as defg.mat and the lambda z as lamz.mat. Lastly, the code plots the Exx and Eyy. By doing this, the user can see if the tracking of the points was smooth. If the lines are not smooth, images need to be reanalyzed with a different pixel number in the automarkertrack.m function.
MATLAB code Biaxial_3 then creates the excel file rawdata.xlsx that contains the x and y force values from the csv file acquired from the system and the deformation calculated from MATLAB code Biaxial_2 for the stretch being analyzed. The user runs the program and is asked to input the number stretches done and the stretch that will be analyzed. Like in MATLAB code Biaxial_1, the code sorts and indexes the force according to each stretch from the csv file. It then creates matrices of the x and y force of the loading curve of the analyzed stretch and their averages. It then reads the excel sheet deformresults.xlsx and creates matrices of the deformation gradient in the x and y deformation of the loading curve. It then creates an excel file (rawdata.xlsx) containing the force and deformation gradients.

The last MATLAB code (Biaxial_4) calculates the x and y tension as well as the 1\textsuperscript{st} and 2\textsuperscript{nd} Piola-Kirchhoff (P-K) stress. The excel file rawdata.xlsx is read and the x and y force and the green strain in the circumferential and radial direction are loaded. The user is then prompted to input the thickness of the sample. This can be measured by placing a portion of the sample tested into OCT, sectioning samples, and then measuring the thickness in ImageJ. The width is assumed to be 1 cm. The 1\textsuperscript{st} P-K stress is calculated by dividing the force by the area of the sample. Defg.mat and lamz.mat is loaded, the deformation gradient at F11 and F22 is extracted from defg.mat. The 2\textsuperscript{nd} P-K stress is calculated by multiplying the inverse of F by 1\textsuperscript{st} P-K stress. Areal strain is calculated by multiplied the E in the circumferential direction by E in the radial direction. The anisotropy index is calculated by dividing the max E in the circumferential direction by E in the radial direction. In the command window, the maximum areal strain and the anisotropy index will be shown. It then creates an excel file (plotdata.xlsx) containing E, S, and P in both the circumferential and radial direction and the areal strain.
4.2.4 Quantifying mechanical properties

Using the equibiaxial data, various mechanical properties can be quantified. As biological soft tissues have non-linear mechanical properties, we quantified the low and high tangent moduli as follows. The low tangent modulus is the stiffness in the toe region that represents the uncrimping of the collagen fibers in the tissue. The high tangent modulus is the stiffness in the linear region that represents the stretching of tissue collagen fibers. The best fit line ($y=mx+b$) was calculated these two tangent moduli in the circumferential and radial directions, where the slope ($m$) of each line was the tangent modulus (74). Extensibility was calculated by the x-intercept of the high tangent modules. The intersection on the x-axis was calculated by using the high tangent modulus best fit line ($x= (y-b) ÷ m$) (Figure 4-3) (74).

![Figure 4-3. 2nd P-K stress vs Green strain graph indicating (A) high and low tangent modulus and (B) extensibility.](image)

Areal strain was calculated by multiplying the maximum Green strain in the circumferential and radial direction ($E_{CIRC,MAX} * E_{RAD,MAX}$). Strain anisotropy index was calculated by dividing the maximum Green strain in the circumferential and radial direction ($E_{CIRC,MAX} ÷ E_{RAD,MAX}$) (Figure 4-4). A strain anisotropy index close to 1 indicated a more isotropic tissue while a value deviating from 1 indicated increasing tissue anisotropy.
Figure 4-4. $2^{nd}$ P-K stress vs Green strain graph indicating maximum Green strain used to calculate areal strain and strain anisotropy.

4.2.5 Statistical methods

Results are reported as mean with standard error. Data was first analyzed for normality using the Shapiro-Wilk normality test. For the enzyme inhibition, a one way ANOVA was run using multiple comparisons versus the diseased (ang-II) state using the Dunnett’s Method. For the receptor inhibition, a two way ANOVA was run using all pairwise multiple comparison followed by the Tukey post hoc test for normally distributed data. Analyses were performed using Sigma Plot. A $p$ value of less than 0.05 was used to indicate statistical significance.

4.3 Results

A total of 6-8 samples were tested for each treatment group. After testing, some samples were excluded from the analysis (Table 4-1). See Appendix C for images of samples during testing (Figure 0-1). Reasons to exclude samples:

- Steel wires ripped off during testing
- Steel wires were not placed properly causing the sample to be loaded unevenly
- Sample believed to be mounted backwards (circumferential in y axis and radial in x axis)
- Sample completely tore and all tension ratio tests were not completed
**Table 4-1.** Number of samples tested and number of samples excluded and included in analysis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of samples tested</th>
<th>Number of samples excluded</th>
<th>Number of samples analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Ang-I</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Ang-II</td>
<td>8</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Ang-I+Quinaprilat</td>
<td>7</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Ang-I+Chymostatin</td>
<td>6</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Ang-I+Losartan</td>
<td>7</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Ang-II+Losartan</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ang-I+PD123,319</td>
<td>7</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Ang-II+PD123,319</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

Equibiaxial stress-strain data allows the quantification of the mechanical properties of the aortic valve leaflets. The max areal strain (ASmax), anisotropy index (AI), low (TM_L) and high (TM_H) tangent modulus for the circumferential and radial direction, and extensibility for the circumferential and radial direction was calculated for each individual sample and were then averaged by treatment groups (**Table 4-2**).
Table 4-2. Max areal strain ($AS_{\text{max}}$), anisotropy index (AI), low (TM$_L$) and high (TM$_H$) tangent modulus for the circumferential and radial direction, and extensibility for the circumferential and radial direction for the average (ave) of each individual specimens and standard error (SEM).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$AS_{\text{max}}$</th>
<th>AI</th>
<th>Circ TM$_L$</th>
<th>Circ TM$_H$</th>
<th>Rad TM$_L$</th>
<th>Rad TM$_H$</th>
<th>Circ Ext</th>
<th>Rad Ext</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Ave 0.097</td>
<td>0.525</td>
<td>54.8</td>
<td>3107.0</td>
<td>22.5</td>
<td>1102.8</td>
<td>0.176</td>
<td>0.338</td>
</tr>
<tr>
<td></td>
<td>SEM 0.014</td>
<td>0.084</td>
<td>10.1</td>
<td>523.5</td>
<td>5.9</td>
<td>71.0</td>
<td>0.024</td>
<td>0.029</td>
</tr>
<tr>
<td>Ang-I</td>
<td>Ave 0.115</td>
<td>0.581</td>
<td>41.8</td>
<td>2522.0</td>
<td>20.6</td>
<td>1275.5</td>
<td>0.201</td>
<td>0.379</td>
</tr>
<tr>
<td></td>
<td>SEM 0.017</td>
<td>0.084</td>
<td>8.1</td>
<td>276.3</td>
<td>5.5</td>
<td>169.4</td>
<td>0.018</td>
<td>0.057</td>
</tr>
<tr>
<td>Ang-II</td>
<td>Ave 0.083</td>
<td>0.463</td>
<td>56.3</td>
<td>3604.2</td>
<td>19.1</td>
<td>1344.7</td>
<td>0.138</td>
<td>0.337</td>
</tr>
<tr>
<td></td>
<td>SEM 0.018</td>
<td>0.083</td>
<td>17.2</td>
<td>750.0</td>
<td>3.1</td>
<td>228.2</td>
<td>0.036</td>
<td>0.035</td>
</tr>
<tr>
<td>Ang-I+ Quinaprilat</td>
<td>Ave 0.098</td>
<td>0.513</td>
<td>56.0</td>
<td>2986.4</td>
<td>21.5</td>
<td>1146.2</td>
<td>0.168</td>
<td>0.375</td>
</tr>
<tr>
<td></td>
<td>SEM 0.011</td>
<td>0.119</td>
<td>7.8</td>
<td>778.3</td>
<td>4.3</td>
<td>192.0</td>
<td>0.013</td>
<td>0.054</td>
</tr>
<tr>
<td>Ang-I+ Chymostatin</td>
<td>Ave 0.083</td>
<td>0.507</td>
<td>53.8</td>
<td>3268.5</td>
<td>33.9</td>
<td>1295.8</td>
<td>0.163</td>
<td>0.325</td>
</tr>
<tr>
<td></td>
<td>SEM 0.013</td>
<td>0.084</td>
<td>10.7</td>
<td>277.3</td>
<td>4.4</td>
<td>193.5</td>
<td>0.025</td>
<td>0.032</td>
</tr>
<tr>
<td>Ang-I+ Losartan</td>
<td>Ave 0.110</td>
<td>0.385</td>
<td>37.7</td>
<td>2690.7</td>
<td>12.6</td>
<td>1101.0</td>
<td>0.158</td>
<td>0.440</td>
</tr>
<tr>
<td></td>
<td>SEM 0.025</td>
<td>0.055</td>
<td>6.5</td>
<td>643.6</td>
<td>1.9</td>
<td>283.1</td>
<td>0.022</td>
<td>0.044</td>
</tr>
<tr>
<td>Ang-II+ Losartan</td>
<td>Ave 0.076</td>
<td>0.321</td>
<td>42.1</td>
<td>5147.7</td>
<td>16.7</td>
<td>1079.2</td>
<td>0.126</td>
<td>0.395</td>
</tr>
<tr>
<td></td>
<td>SEM 0.011</td>
<td>0.054</td>
<td>8.4</td>
<td>1145.7</td>
<td>5.0</td>
<td>78.0</td>
<td>0.016</td>
<td>0.044</td>
</tr>
<tr>
<td>Ang-I+ PD123,319</td>
<td>Ave 0.094</td>
<td>0.361</td>
<td>64.6</td>
<td>5214.7</td>
<td>19.7</td>
<td>1198.4</td>
<td>0.154</td>
<td>0.433</td>
</tr>
<tr>
<td></td>
<td>SEM 0.012</td>
<td>0.062</td>
<td>11.0</td>
<td>496.6</td>
<td>4.8</td>
<td>116.9</td>
<td>0.019</td>
<td>0.048</td>
</tr>
<tr>
<td>Ang-II+ PD123,319</td>
<td>Ave 0.094</td>
<td>0.455</td>
<td>60.9</td>
<td>3388.1</td>
<td>17.3</td>
<td>1415.0</td>
<td>0.158</td>
<td>0.398</td>
</tr>
<tr>
<td></td>
<td>SEM 0.012</td>
<td>0.072</td>
<td>6.4</td>
<td>463.9</td>
<td>2.0</td>
<td>319.9</td>
<td>0.014</td>
<td>0.035</td>
</tr>
</tbody>
</table>

4.3.1 Areal strain and strain anisotropy index

The max areal strain ($AS_{\text{max}}$) and strain anisotropy (AI) were calculated from the maximum Green strain (E) in the equibiaxial loading condition. The ang-I treated group had a higher $AS_{\text{max}}$ than the control while the ang-II treated group had a lower $AS_{\text{max}}$ than the control (Figure 4-5a,b). When ang-I was supplemented with an ACE inhibitor, it had a similar $AS_{\text{max}}$ to the control and lower $AS_{\text{max}}$ than the ang-I treated group. When ang-I was supplemented with a chymase inhibitor, it had a lower $AS_{\text{max}}$ than the control and ang-I treated group (Figure 4-5a).
Ang-I supplemented with an AT-1R antagonist had similar $A_{\text{max}}$ than the ang-I treated group and higher $A_{\text{max}}$ than the control. When ang-II was supplemented with an AT-1R antagonist, it had a lower $A_{\text{max}}$ than the control and ang-II treated group. Ang-I supplemented with an AT-2R antagonist had a similar $A_{\text{max}}$ than the control and lower $A_{\text{max}}$ than the ang-I treated group. Ang-II supplemented with an AT-2R antagonist had a higher $A_{\text{max}}$ than the ang-II treated group and a lower $A_{\text{max}}$ than the control (Figure 4-5b). Ang-I treated group had a higher AI than the control while the ang-II treated group had a lower AI than the control (Figure 4-5c,d). Ang-I supplemented with an ACE and chymase inhibitor had a lower AI than the control and ang-I treated group (Figure 4-5c). When ang-I and ang-II were supplemented with an AT-1R antagonist, it had a lower AI than the control, ang-I, and ang-II treated groups. When ang-I was supplemented with an AT-2R antagonist, it had a lower AI than the control and ang-I treated group. When ang-II was supplemented with an AT-2R antagonist, it had a lower AI than the control but a similar AI than the ang-II treated group (Figure 4-5d).
4.3.2 Tangent modulus

The low tangent modulus (TM$_L$) is the stiffness in the toe region where the fibers are “un-crimping”. In the circumferential region, the control and ang-II had a similar TM$_L$ whereas the TM$_L$ for samples treated with ang-I was slightly less (Figure 4-6a,b). When treated with ang-I supplemented with the ACE or chymase inhibitors, the TM$_L$ was similar to that of the control (Figure 4-6a). When treated with ang-I or ang-II supplemented with the AT-1R inhibitor, the samples had a lower TM$_L$ than when treated with ang-I or ang-II alone implying that by inhibiting binding of ang-II to AT-1R the samples are less stiff. When treated with ang-I or ang-II supplemented with the AT-2R inhibitor, the samples had a higher TM$_L$ than when treated with ang-I or ang-II alone. This implies that by inhibiting AT-2R and allowing more ang-II to bind to AT-1R, the leaflets are stiffer (Figure 4-6b). In the radial direction, the control, ang-I, and ang-II
had a similar TM<sub>L</sub> (Figure 4-6c,d). When treated with ang-I supplemented with the ACE inhibitor, the TM<sub>L</sub> was similar to that of the control. However, ang-I supplemented with the chymase inhibitor had a higher TM<sub>L</sub> (Figure 4-6c). When treated with ang-I or ang-II supplemented with the AT-1R inhibitor, the samples had a lower TM<sub>L</sub> than when treated with ang-I or ang-II alone. When treated with ang-I or ang-II supplemented with the AT-2R inhibitor, the samples had a similar TM<sub>L</sub> than when treated with ang-I or ang-II alone (Figure 4-6d).

The high tangent modulus (TM<sub>H</sub>) is the stiffness in the linear region where the fibers have been “uncrimpled” and are now being stretched. In the circumferential direction, ang-I had a lower tangent modulus than the control, and ang-II had a higher TM<sub>H</sub> than the control (Figure 4-6e,f). The TM<sub>H</sub> of samples treated with ang-I supplemented with ACE and chymase inhibitor was similar to that of the control (Figure 4-6e). Tissue treated with ang-I supplemented with the AT-1R inhibitor had a lower TM<sub>H</sub>, and tissue treated with ang-I supplemented with AT-2R inhibitor had a higher TM<sub>H</sub>. On the contrary, tissue treated with ang-I supplemented with the AT-1R inhibitor had a higher TM<sub>H</sub>, and tissue treated with ang-I supplemented with AT-2R inhibitor had a lower TM<sub>H</sub> (Figure 4-6f). In the radial direction, ang-I treated tissue had a higher TM<sub>H</sub> than the control, and ang-II treated tissue had a higher TM<sub>H</sub> than both the control and ang-I (Figure 4-6g,h). When treated with ang-I supplemented with the ACE inhibitor, the TM<sub>H</sub> was similar to that of the control. However, tissue treated with ang-I supplemented with chymase inhibitor had a similar TM<sub>H</sub> than when treated with ang-I alone (Figure 4-6g). When treated with ang-I or ang-II supplemented with the AT-2R inhibitor, the TM<sub>H</sub> was similar to that of the control. When treated with ang-I or ang-II supplemented with the AT-2R inhibitor, the TM<sub>H</sub> was similar to those treated with ang-I or ang-II alone (Figure 4-6h).
4.3.3 Extensibility

In the circumferential direction, ang-I had a higher extensibility than the control, and ang-II had a lower extensibility than the control (Figure 4-7a,b). When treated with ang-I supplemented with the ACE or chymase inhibitors, the extensibility was similar to that of the control (Figure 4-7a). When ang-I and ang-II were supplemented with an AT-1R antagonist, they had a lower extensibility than the control, ang-I, and ang-II treated group. When ang-I was supplemented with an AT-2R antagonist, it had a lower extensibility than the control and ang-I treated group. Ang-II supplemented with an AT-2R antagonist had a lower extensibility than the control but a higher extensibility than the ang-II treated group (Figure 4-7b). In the radial direction, ang-II treated group had a similar extensibility than the control, but the ang-I treated group had a greater extensibility than the control (Figure 4-7c,d). Ang-I supplemented with an ACE inhibitor had a similar extensibility than the ang-I treated group. When ang-I was
supplemented with a chymase inhibitor, it had a similar extensibility than the control (Figure 4-7c). When ang-I and ang-II were supplemented with an AT-1R inhibitor, they had a greater extensibility than the control, ang-I, and ang-II treated group. Ang-I and ang-II supplemented with an AT-2R inhibitor had a higher extensibility than the control, ang-I, and ang-II treated group (Figure 4-7d).

![Figure 4-7](image)

*Figure 4-7. The extensibility in the circumferential direction for (A) enzyme and (B) receptor inhibitors and in the radial direction for (C) enzyme and (D) receptor inhibitors. Red dotted line is the untreated, native leaflets.*

### 4.4 Discussion

When the anisotropy index (AI) was calculated with the equibiaxial data, all treatment groups had an AI of less than 0.6. An AI close to 1 means that a sample is isotropic so our results showed that all samples were anisotropic as the AI was less than 1. The treatment groups where ang-I and ang-II were supplemented with an AT-1R had the lowest calculated AI. This implies
that when the ability for ang-II to bind to AT-1R is mitigated, the anisotropic qualities of the tissue increases. Ang-II supplemented with an AT-2R antagonist had a similar AI than the ang-II treated groups. This indicates that the effects seen in the AI of ang-II treatment is due to the binding of ang-II to AT-1R.

The enzyme inhibited groups showed a similar tangent modulus in the toe and linear region in the circumferential and radial direction with the exception of the chymase inhibitor in the toe and linear region in the radial direction. This implies that by preventing ang-I to convert to ang-II the stiffness is similar to that of the non-treated samples. Though ang-I and ang-II treated groups did not seem to have a different tangent modulus in the circumferential direction or in the toe region in the radial direction, in the linear region in the radial direction ang-I and ang-II had a higher tangent modulus. This implies that in the cross-fiber direction the stiffness of the collagen fibers does increase. In the toe region in the circumferential and radial direction as well as the linear region in the radial direction, ang-I and ang-II supplemented with an AT-1R antagonist had the lowest tangent modulus overall. Only in the linear region in the circumferential direction did ang-II supplemented with AT-1R antagonist have a greater tangent modulus, however ang-I supplemented with AT-1R antagonist did have a lesser tangent modulus in that region. This implies that by inhibiting the ability for ang-II to bind to AT-1R the stiffness decreases. This is further verified in the AT-2R inhibited treatment groups. Ang-I and ang-II supplemented with the AT-2R antagonist had a higher tangent modulus in the toe and linear region in the circumferential and radial direction with the exception of ang-II supplemented with AT-2R antagonist in the linear region in the circumferential direction. By inhibiting the ability for ang-II to bind to AT-2R, ang-II will bind more to AT-1R increasing the stiffness of the sample. There was a high variation in the extensibility, and it seems to be highly unreliable.
because small changes in the high TM fit will result in wide changes in where the line cuts the x-axis, thereby affecting extensibility.

Tissue being treated with ang-I and ang-II may not have shown a trend in the change of mechanical behaviors as this was a short term treatment. Though ang-II is more prone to bind to AT-1R, the amount of time may not have been sufficient to increase the binding of AT-1R over AT-2R. However, limiting the ability to bind to just one receptor did show a trend in mechanical behavior.

In the next chapter, we will use the multiple tension loading ratios to develop three non-linear phenomenological constitutive models to characterize the mechanical behavior of aortic valve tissues pretreated with RAS mediators. In this aim, all these measurements are very dependent on fitting to inherently non-linear data, and the constitutive models we’ll use are non-linear models that fit the data properly.
CHAPTER 5 SPECIFIC AIM 2

Utilize phenomenological constitutive models to characterize the biomechanical changes that occur due to RAS mediators on aortic valves

5.1 Introduction

Phenomenological constitutive models can be used to quantify the anisotropic and nonlinear material properties of soft tissue such as aortic valve leaflets (75). They can account for the regional variation and the properties of the leaflet layers, and the stress environment the cells undergo can be predicted with the appropriate model (76). In incompressible materials such as isotropic rubber materials, they undergo finite deformation in which a two-dimensional stress-state is sufficient to develop constitutive equations such as the Mooney-Rivlin (M-R) (77, 78). However, biological tissue require more rigorous models due to their complex mechanical behavior (77). Viscoelastic behavior of materials is discussed through mechanical models in terms of how a material behaves when stress or strain is applied (79). Isotropic models such as M-R have been modified to be used for anisotropic material by adding an additional anisotropic term (80). A more commonly viscoelastic anisotropic model used for valve leaflets is the Fung-type model (75, 81). Material constants derived from constitutive models are important for generating computational simulations of tissue deformation. Simulations can be performed using specimen-specific constants, however, having material constants that represent a generic (average) response is favorable (75).

This aim focuses on selecting an appropriate constitutive model that mimics the biomechanical changes that occur in aortic valves due to RAS mediators. In addition, material constants will be derived from an average response to simulate the generic mechanical behavior.
Mechanical properties will be assessed through the anisotropy index calculated through material constants and the stress-strain response graphs from the average model.

5.2 Methods

MATLAB codes can be found in Appendix B. Prior to running the MATLAB codes, excels sheets need to be created with the appropriate information. Refer to Appendix B as it is important to create the excel file in the correct order as MATLAB codes load the data accordingly.

5.2.1 Average model

The average stress-strain response can be calculated by using an average model based on identical tension states (75). The user is asked to first choose the file containing the thickness of each sample and then the file containing the x and y-force and E11 and E22. The MATLAB code will then extract the information and create an individual matrix for each. The x and y-tension is calculated by dividing the force by the width which is assumed to be 1 cm. The average tension ($T_{avg,ii}$) for x and y is calculated by averaging the individual x and y tensions. The same is done to calculate the average green strain ($E_{avg,ii}$) for the x (circumferential, E11) and y (radial, E22) directions. The average 1st P-K stress ($P_{avg,ii}$) is calculated with Eq. (5.1).

$$p_{avg,ii} = \frac{T_{avg,ii}}{n} \sum_{i=1}^{n} \frac{1}{h_i}$$

(5.1)

$n$ is the number of samples and $h_i$ is the thickness of the individual leaflets. The average deformation gradient ($F_{avg,ii}$) is calculated using Eq. (5.2) assuming negligible shear deformation.

The average 2nd P-K stress ($S_{avg,ii}$) is given by Eq. (5.3).

$$F_{avg,ii} = \sqrt{2P_{avg,ii} + 1}$$

(5.2)
The average anisotropy index $A_{\text{avg}}$ is calculated by dividing the max $E_{\text{avg},11}$ and $E_{\text{avg},22}$.

The code then exports two excel files. AveragePlotData.xlsx contains the average of E, S, and P for each direction and $A_{\text{avg}}$. AverageEandTandStandardError.xls contains the average E and T and the standard errors for E and T in each direction. The code then creates two figures. Figure 1 is $S_{\text{avg},ii}$ vs $E_{\text{avg},ii}$ including the standard error bars, and figure 2 is $S_{\text{avg},ii}$ vs $E_{\text{avg},ii}$ without the standard error bars.

### 5.2.2 Mooney-Rivlin model

Two modified Mooney-Rivlin (M-R) constitutive models were used: an isotropic and an anisotropic model. The strain energy equation function for the isotropic M-R model is given by Eq (5.4), and the strain energy ($W$) equation function for the anisotropic M-R model is given by Eq (5.5) (80).

$$W=c_1 (I_1-3) + c_2 (I_2-3) + D_1 \left[ \exp \left( D_2 (I_1 - 3) \right) - 1 \right]$$  \hspace{1cm} (5.4)

$$W=c_1 (I_1-3) + D_1 \left[ \exp \left( D_2 (I_1 - 3) \right) - 1 \right] + \left( k_1/2k_2 \right) \left[ \exp \left[ k_2 (I_4 - 1)^2 \right] - 1 \right]$$  \hspace{1cm} (5.5)

$W$ is a function of the strain invariants (I) can be calculated using Eq. (5.6) (78).

$$I_1=\text{tr} (B)=\lambda_1^2+\lambda_2^2+\lambda_3^2$$  \hspace{1cm} (5.6)

$$I_2=0.5 \left[ (\text{tr}B)^2-\text{tr}(B^2) \right]=\lambda_1^2 \lambda_2^2+\lambda_2^2 \lambda_3^2+\lambda_3^2 \lambda_1^2$$

$$I_4=C_{ij}(n_c)(n_c)_{ij}$$

$\lambda_1$, $\lambda_2$, and $\lambda_3$ are the principal stretches and the left Cauchy-Green tensor ($B$) can be calculated by $B=FF^T$. $n_c$ refers to the principle direction of the material fibers. The standard constitutive model for an incompressible, isotropic, elastic material can be determined with Eq. (5.7) (78).

$$\sigma = -p 1 + 2 \frac{dW}{dI_1} B - 2 \frac{dW}{dI_2} B^{-1}$$  \hspace{1cm} (5.7)
\( p \) is a hydrostatic pressure term associated with the incompressibility constraints and \( \sigma \) is Cauchy stress. For this study, the stress for the models needs to be in 2nd P-K stress (S) and relative to Green strain (E). The relationship between \( \sigma \), \( P \), and \( S \) are the following (82):

\[
\sigma = \frac{1}{J}FP
\]

\[
P=FS
\]

\( J \) is the Jacobian and \( F \) is the deformation gradient. \( J \) is a measurement of the volume change produced by the deformation. We assumed that there is no change in volume so \( J=1 \) hence \( \sigma \) relates to \( S \) by Eq. (5.9).

\[
\sigma = F^2 S
\]

The relationship between \( E \) and \( \lambda_1, \lambda_2, \) and \( \lambda_3 \) can be calculated using \( F \) with Eq. (5.11) (82).

\[
F = \begin{bmatrix} \lambda_1 & 0 & 0 \\ 0 & \lambda_2 & 0 \\ 0 & 0 & \lambda_3 \end{bmatrix}
\]

\[
E = 0.5(F^T F - I)
\]

Using Eq. (5.9) the following can be said about \( S \) in the circumferential and radial direction:

\[
S_{11} = \sigma / \lambda_1^2
\]

\[
S_{22} = \sigma / \lambda_2^2
\]

Using Eq. (5.11), the following can be said about \( \lambda \) in terms of \( E \):

\[
\lambda_1^2 = 2E_{11} + 1
\]

\[
\lambda_2^2 = 2E_{22} + 1
\]

\[
\lambda_3^2 = 2E_{33} + 1
\]

Using the strain energy equation for the isotropic and anisotropic modified M-R, Eq. (5.4) and Eq. (5.5) respectively, \( S \) in the circumferential and radial direction were calculated in terms of \( E \). Note: \( E_{33} \) was assumed to be 0 from deformation results in Aim 1.
Isotropic modified M-R:

\[ S_{11} = [2c_2 - 2 (c_1 + D_1 [D_2 \exp(D_2(2E_{11} + 2E_{22} + 3) - 3D_2))] + 2(2E_{11} + 1) (c_1 + D_1[D_2\exp(D_2(2E_{11} + 2E_{22} + 3) - 3D_2))] - 2c_2 (2E_{11} + 1)^{-1}] \div (E_{11} + 1) \]  

(5.14)

\[ S_{22} = [2c_2 - 2 (c_1 + D_1 [D_2 \exp(D_2(2E_{11} + 2E_{22} + 3) - 3D_2))] + 2(2E_{22} + 1) (c_1 + D_1[D_2\exp(D_2(2E_{11} + 2E_{22} + 3) - 3D_2))] - 2c_2 (2E_{22} + 1)^{-1}] \div (E_{11} + 1) \]  

(5.15)

Anisotropic modified M-R:

\[ S_{11} = [4E_{11} (c_1 + D_1D_2 \exp(2E_{11} + 2E_{22})) + 4k_1E_{11} (2E_{11} + 1) \exp(k_2(2E_{11})^2)] \div (2E_{11} + 1) \]  

(5.16)

\[ S_{22} = [4E_{22} (c_1 + D_1D_2 \exp(2E_{11} + 2E_{22}))] \div (2E_{22} + 1) \]  

(5.17)

The constants for the M-R constitutive model were calculated using MATLAB, ModifiedMooneyRivlin_Isotropic.m for the modified M-R isotropic model and MATLAB code ModifiedMooneyRivlin_Anisotropic.m for the modified M-R anisotropic model. The MATLAB code uses the objective function to calculate the sum of squares of the residuals given by Eq. (5.18) (82).

\[ y^2 = \sum_{i=1}^{n} (\sigma_{11} - \sigma_{11}^m)^2 + (\sigma_{22} - \sigma_{22}^m)^2 \]  

(5.18)

In the isotropic model, it used Eq. (5.14) and Eq.(5.15), and in the anisotropic model it used Eq. (5.16) and Eq. (5.17). The minimization of the objective function was done by using the function fmincon. In the anisotropic model, D_2 was set to 2 without appreciably reducing the goodness of fit (82). The code then calculates the constants and total R^2. The isotropic MATLAB code exports an excel sheet (Modified M-R (Isotropic) Constants.xlsx) containing the c_1, c_2, D_1, and D_2 constants and the R^2 combined. The anisotropic MATLAB code exports an excel sheet (Modified M-R (Anisotropic) Constants.xlsx) containing the c_1, D_1, D_2, k_1, and k_2 constants and the R^2 combined.
Using the constants, it generates values for plotting the model fit for each tension load ratio using the experimental values $E_{11}$ and $E_{22}$ in Eq. (5.21) and Eq (5.22). It plots two figures using the model fit. Figure 1 plots the individual ratios (5 subplots) with the circumferential and radial direction in one graph. Figure 2 plots all the ratios in either the circumferential or radial direction (2 subplots).

5.2.3 Fung Type model

The strain energy density ($W$) function of the fung-type model utilized is given by Eq. (5.19).

$$W=0.5c \left(e^{Q}-1\right),$$  \hspace{1cm} (5.19)

where $Q=c_1E_{11}^2+c_2E_{22}^2+2c_3E_{11}E_{22}$. The second P-K stress tensor ($S_{ij}$) is evaluated by Eq. (5.20).

$$S_{ij}=\frac{dW}{dE_{ij}},$$  \hspace{1cm} (5.20)

$E_{ij}$ are the components of the Green strain tensor and $i$ and $j$ are dummy indices (75). $S_{11}$ and $S_{22}$, circumferential and radial directions respectively, were evaluated using Eq. (5.21) and Eq (5.22).

$$S_{11}=0.5c*\exp( c_1E_{11}^2+ c_2E_{22}^2+2 c_3E_{11}E_{22})*(2c_1 E_{11}+2c_3E_{22})$$  \hspace{1cm} (5.21)

$$S_{22}=0.5c*\exp( c_1E_{11}^2+ c_2E_{22}^2+2 c_3E_{11}E_{22})*(2c_2 E_{22}+2c_3E_{11})$$  \hspace{1cm} (5.22)

The constants for the Fung-type constitutive model were calculated using MATLAB code FungModelFit_EnergyPlot.m. The MATLAB code uses the objective function to calculate the sum of squares of the residuals using Eq. (5.21) and Eq. (5.22) for each tension load ratio. The minimization of the objective function was done by using the function fmincon which was performed with the following constraints (81):
c > 0

\[ c_1 > |c_3| \]

\[ c_2 > |c_3| \]

The code calculates the optimal c, c_1, c_2, and c_3 constants, and calculates the total R^2.

Using the constants, a non-linear anisotropy index (AI) was calculated using Eq. (5.23) (75).

\[
AI = \min \left( \frac{c_1 + c_3}{c_2 + c_3}, \frac{c_2 + c_3}{c_1 + c_3} \right)
\]  

(5.23)

It then exports an excel sheet (Fung-type Constants.xlsx) containing the c, c_1, c_2, and c_3 constants, the condition number, R^2 combined, and the AI. Using the constants, it generates values for plotting the model fit for each tension load ratio using the experimental values E11 and E22 in Eq. (5.21) and Eq (5.22). It plots two figures using the model fit. Figure 1 plots the individual ratios (5 subplots) with the circumferential and radial direction in one graph. Figure 2 plots all the ratios in either the circumferential or radial direction (2 subplots).

In addition to calculating the Fung-type constants, the MATLAB code also generates a strain energy contour plot. Contour plots are used to show that the material constants fulfill the positive definiteness requirement of elasticity tensor by satisfying the strain energy convexity constraints. Contour plots are generated by graphing E11 and E22 in a range from -1.5 to 1.5 into the strain energy Eq. (5.19) using the material constants. The contours are plotted at W= 0.01, 0.5, 3, 10, and 30 (75).

**5.2.4 Average stress-strain graph analysis**

By graphing the 2nd P-K Stress vs green strain of the various tension ratios in the circumferential and radial direction, there are mechanical properties that can be observed. (Figure 5-1). First is the mechanical anisotropy which shows if samples have a different stress-strain behavior in the circumferential and radial direction. The native valve is typically highly
anisotropic and any trend towards less anisotropy suggest pathological changes. An anisotropic sample will show a spacing between the circumferential and radial curves. The second is the cross coupling of the fibers which is where the stress in one direction affects the mechanical behavior of the orthogonal direction. If we look at circumferential curves, the equibiaxial, black dots, is in the middle as it was stretched equally in both directions. The green diamonds are in which it was stretch 3/4 in the circumferential direction and full tension in the radial direction, and the blue diamond’s half the tension as the radial direction. As it can be seen, the sample underwent less strain in the circumferential direction in comparison, but in the radial direction (grey diamond) corresponding to that stretch, it underwent the most strain. This means that the strain in each direction is affected by how much stress is applied in the perpendicular direction. This is the cross-coupling affect and can be seen by the spreading out of the stress strain curves.

![Diagram](image)

**Figure 5-1.** (A) Aortic valve leaflet (73) showing circumferential and radial direction. (B) 2nd P-K stress vs Green strain graph for tension ratios 1:1, 1:0.75, 0.75:1, 1:0.50, 0.50:1

### 5.2.5 Statistical methods

Results are reported as mean with standard error. Data was first analyzed for normality using the Shapiro-Wilk normality test. For the enzyme inhibition, a one way ANOVA was run using multiple comparisons versus the diseased (ang-II) state using the Dunnett’s Method. For the receptor inhibition, a two way ANOVA was run using all pairwise multiple comparison
followed by the Tukey post hoc test for normally distributed data. Analyses were performed using Sigma Plot. A $p$ value of less than 0.05 was used to indicate statistical significance.

### 5.3 Results

The material constants for the modified M-R isotropic and anisotropic constitutive model and the Fung-type model were calculated for each individual sample using the green strain acquired from the experimental data acquired during biaxial testing. In addition, the material constants were calculated for the average stress-strain response given from the average model.

A total of 6-8 samples were tested for each treatment group. After testing, some samples were excluded from the analysis (Table 5-1). See Appendix C for images of samples during testing.

Reasons to exclude samples:

- Steel wires ripped off during testing
- Steel wires were not placed properly causing the sample to be loaded unevenly
- Sample believed to be mounted backwards (circumferential in $y$ axis and radial in $x$ axis)
- Sample completely tore and all tension ratio tests were not completed

### Table 5-1. Number of samples tested and number of samples excluded and included in analysis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of samples tested</th>
<th>Number of samples excluded</th>
<th>Number of samples analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Ang-I</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Ang-II</td>
<td>8</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Ang-I+Quinaprilat</td>
<td>7</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Ang-I+Chymostatin</td>
<td>6</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Ang-I+Losartan</td>
<td>7</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Ang-II+Losartan</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ang-I+PD123,319</td>
<td>7</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Ang-II+PD123,319</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>
5.3.1 Average model

Mechanical testing in the circumferential and radial axes consisted of the following tension ratios with a maximum tension of 90 N/m: \( T_{\text{circ}}:T_{\text{rad}} - 1:1, 1:0.75, 1:0.5, 0.75:1, 0.5:1 \). In the circumferential direction, most tension ratios and treatment groups had a low standard error. However, in the radial direction, some tension ratios had a higher standard error suggesting greater variation between samples in each treatment group in the cross fiber direction (Figure 5-2).
Figure 5-2. Average response based on identical tension states for each tension ratio with a Green strain standard error of each treatment group.
5.3.2 Model Fit

**Modified M-R isotropic model**

The material constants for each individual specimen varied greatly as can be seen in Table 5-2. For example, in the control, the D1 constant was 0.14 in one sample whereas it was -1144.73 in another sample. When the D1 constant was averaged in the control treatment group, it had a value of 45.23 with a standard error of 398.71. This trend is seen in the majority material constants in treatment groups. This showed that by averaging the individual constants in each treatment group an accurate finite constitutive model would not be possible. Table 5-2 also shows that the \(R^2\) is very low in all samples and in their average implying that this model was not a good fit for the data.

**Table 5-2.** Material constants c1, c2, D1, and D2 for the Modified Mooney-Rivlin isotropic constitutive model and the \(R^2\) of the fit for individual specimens by fitting the experimental data.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample No.</th>
<th>c1</th>
<th>c2</th>
<th>D1</th>
<th>D2</th>
<th>(R^2)</th>
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<td>45.23</td>
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<td>c2</td>
<td>D1</td>
<td>D2</td>
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</table>
In (Table 5-3), the material constants were derived from the average model. The material constants from the average model are very different than those from when the material constants of each sample were averaged. The R² in all treatment group was less than 0.50 which indicates a poor fit. This is expected as valve leaflets are anisotropic material and not isotropic material.

Table 5-3 Material constants c1, c2, D1, and D2 for the Modified Mooney-Rivlin isotropic constitutive model and the R² of the fit for the average response based on identical tensions.

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<tr>
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<th>c2</th>
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<th>D2</th>
<th>R²</th>
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<td>-109.37</td>
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</table>

When the model fit was plotted with the experimental data, it can be seen that it does not follow the shape of the stress strain curve (Figure 5-3). The fit for the different tension loading...
ratios are all similar to one another meaning that there is no spreading between the loading ratios. The max 2\textsuperscript{nd} P-K stress of the model fit in the circumferential and radial direction was half if not much less than that of the experimental data.

\textbf{Figure 5-3.} The Modified Mooney-Rivlin isotropic constitutive model fit along the experimental data in the circumferential and radial direction for the average response of each treatment
Modified M-R anisotropic model

The material constants for each individual specimen did not vary as drastically in the M-R anisotropic model as they did in the isotropic model (Table 5-4). It was only the SEM for the k2 constant that was large. Table 5-4 also shows that the $R^2$ is higher in all samples and in their average in comparison to the isotropic model implying that this modified M-R anisotropic model is a better fit than that of the modified M-R isotropic model.

Table 5-4. Material constants c1, D1, D2, k1, and k2 for the Modified Mooney-Rivlin anisotropic constitutive model and the $R^2$ of the fit for individual specimens by fitting the experimental data

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<th>D2</th>
<th>k1</th>
<th>k2</th>
<th>$R^2$</th>
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</table>
In Table 5-5, the material constants were derived from the average model. The material constants from the average model are still quite different than those from when the material constants of each sample were averaged. It is very clear in the k2 constant where the average of the k2 constants in the control group was 1.54 (Table 5-4) and the k2 for the average model is 7.96. The R² in all treatment group was more than 0.75 which indicates a better fit.

Table 5-5. Material constants c1, D1, D2, k1, and k2 for the Modified Mooney-Rivlin anisotropic constitutive model and the R² of the fit for the average response based on identical tensions.

<table>
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<th>Sample No.</th>
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<th>D2</th>
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<td>-60.96</td>
<td>14.09</td>
<td>2.00</td>
<td>0.93</td>
<td>23.89</td>
<td>0.81</td>
</tr>
<tr>
<td>Ang-II+PD123,319</td>
<td>2</td>
<td>-65.41</td>
<td>15.78</td>
<td>2.00</td>
<td>17.98</td>
<td>19.90</td>
<td>0.77</td>
</tr>
<tr>
<td>Ang-II+PD123,319</td>
<td>3</td>
<td>-97.77</td>
<td>26.65</td>
<td>2.00</td>
<td>10.19</td>
<td>4.73</td>
<td>0.80</td>
</tr>
<tr>
<td>Ang-II+PD123,319</td>
<td>4</td>
<td>-44.89</td>
<td>12.85</td>
<td>2.00</td>
<td>5.23</td>
<td>7.35</td>
<td>0.73</td>
</tr>
<tr>
<td>Ang-II+PD123,319</td>
<td>5</td>
<td>-62.17</td>
<td>16.29</td>
<td>2.00</td>
<td>14.16</td>
<td>7.17</td>
<td>0.66</td>
</tr>
<tr>
<td>Ave</td>
<td>66.24</td>
<td>17.13</td>
<td>2.00</td>
<td>9.70</td>
<td>12.61</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>8.65</td>
<td>2.46</td>
<td>0.00</td>
<td>3.05</td>
<td>3.87</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

When the model fit was plotted with the experimental data, it can be seen that it does not follow the shape of the stress strain curve (Figure 5-4). However, in this model the different tension loading ratios are spreading as seen in the experimental data, suggesting that the modified M-R is able to capture the cross-coupling between the fiber and cross-fiber directions in the samples. The max 2nd P-K stress of the model fit in the circumferential and radial direction
was also closer to that of the experimental data in comparison to the modified M-R isotropic model.

**Figure 5-4.** The Modified Mooney-Rivlin anisotropic constitutive model fit along the experimental data in the circumferential and radial direction for the average response of each treatment
Fung-type model

The Fung-type model provided the best fit. The material constants for each individual samples was consistent as can be seen by the low standard error (Table 5-6). All samples had a much greater R² than either of the modified M-R models.

Table 5-6. Material constants c, c1, c2, and c3 for the Fung-type constitutive model, R² of the fit, and anisotropy index (AI) for individual specimens by fitting the experimental data.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample No.</th>
<th>c(kPa)</th>
<th>c1</th>
<th>c2</th>
<th>c3</th>
<th>R²</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>0.519</td>
<td>9.273</td>
<td>5.750</td>
<td>3.427</td>
<td>0.789</td>
<td>0.723</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.828</td>
<td>13.507</td>
<td>10.866</td>
<td>0.143</td>
<td>0.929</td>
<td>0.807</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.650</td>
<td>15.021</td>
<td>12.657</td>
<td>2.263</td>
<td>0.917</td>
<td>0.863</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.025</td>
<td>23.489</td>
<td>12.816</td>
<td>3.983</td>
<td>0.953</td>
<td>0.612</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.768</td>
<td>36.907</td>
<td>9.974</td>
<td>2.432</td>
<td>0.968</td>
<td>0.315</td>
</tr>
<tr>
<td></td>
<td>Ave</td>
<td>0.758</td>
<td>19.639</td>
<td>10.413</td>
<td>2.450</td>
<td>0.911</td>
<td>0.664</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.085</td>
<td>4.896</td>
<td>1.284</td>
<td>0.658</td>
<td>0.032</td>
<td>0.097</td>
</tr>
<tr>
<td>Ang-I</td>
<td>1</td>
<td>1.020</td>
<td>13.428</td>
<td>6.171</td>
<td>0.634</td>
<td>0.797</td>
<td>0.484</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.446</td>
<td>26.804</td>
<td>8.946</td>
<td>-0.413</td>
<td>0.932</td>
<td>0.323</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.252</td>
<td>22.536</td>
<td>3.497</td>
<td>-1.407</td>
<td>0.773</td>
<td>0.099</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.117</td>
<td>18.514</td>
<td>11.811</td>
<td>-2.554</td>
<td>0.878</td>
<td>0.580</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.834</td>
<td>55.868</td>
<td>22.981</td>
<td>-9.307</td>
<td>0.911</td>
<td>0.294</td>
</tr>
<tr>
<td></td>
<td>Ave</td>
<td>0.934</td>
<td>27.430</td>
<td>10.681</td>
<td>-2.609</td>
<td>0.858</td>
<td>0.356</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.140</td>
<td>7.445</td>
<td>3.373</td>
<td>1.756</td>
<td>0.031</td>
<td>0.083</td>
</tr>
<tr>
<td>Ang-II</td>
<td>1</td>
<td>1.016</td>
<td>19.318</td>
<td>5.132</td>
<td>0.958</td>
<td>0.897</td>
<td>0.300</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.613</td>
<td>15.122</td>
<td>15.580</td>
<td>-1.391</td>
<td>0.830</td>
<td>0.968</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.109</td>
<td>15.167</td>
<td>7.231</td>
<td>-0.482</td>
<td>0.799</td>
<td>0.460</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.190</td>
<td>26.848</td>
<td>9.286</td>
<td>4.411</td>
<td>0.853</td>
<td>0.438</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.303</td>
<td>19.716</td>
<td>17.911</td>
<td>11.475</td>
<td>0.847</td>
<td>0.942</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.145</td>
<td>124.516</td>
<td>31.700</td>
<td>-4.411</td>
<td>0.958</td>
<td>0.227</td>
</tr>
<tr>
<td></td>
<td>Ave</td>
<td>0.896</td>
<td>36.781</td>
<td>14.473</td>
<td>1.760</td>
<td>0.864</td>
<td>0.556</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.293</td>
<td>17.634</td>
<td>3.985</td>
<td>2.274</td>
<td>0.023</td>
<td>0.131</td>
</tr>
<tr>
<td>Ang-I+Quinaprilat</td>
<td>1</td>
<td>1.295</td>
<td>19.452</td>
<td>15.117</td>
<td>-0.026</td>
<td>0.867</td>
<td>0.777</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.410</td>
<td>31.756</td>
<td>9.752</td>
<td>-2.171</td>
<td>0.977</td>
<td>0.256</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.266</td>
<td>43.667</td>
<td>10.297</td>
<td>-0.128</td>
<td>0.920</td>
<td>0.234</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.305</td>
<td>19.559</td>
<td>7.445</td>
<td>-0.453</td>
<td>0.756</td>
<td>0.366</td>
</tr>
<tr>
<td></td>
<td>Ave</td>
<td>0.819</td>
<td>28.609</td>
<td>10.653</td>
<td>-0.695</td>
<td>0.880</td>
<td>0.408</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.279</td>
<td>5.791</td>
<td>1.611</td>
<td>0.501</td>
<td>0.047</td>
<td>0.126</td>
</tr>
</tbody>
</table>
In Table 5-7, the material constants were derived from the average model. The material constants from the average model are very similar than those from when the material constants
of each sample were averaged. The \( R^2 \) in all treatment group was greater than 0.90 indicating that the Fung-type model was an excellent fit for our experimental data.

**Table 5-7.** Material constants c, \( c_1 \), \( c_2 \), and \( c_3 \) for the Fung-type constitutive model, \( R^2 \) of the fit, and anisotropy index (AI) for the average response based on identical tensions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>c(kPa)</th>
<th>( c_1 )</th>
<th>( c_2 )</th>
<th>( c_3 )</th>
<th>( R^2 )</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.729</td>
<td>18.099</td>
<td>10.770</td>
<td>1.528</td>
<td>0.934</td>
<td>0.627</td>
</tr>
<tr>
<td>Ang-II</td>
<td>0.758</td>
<td>24.682</td>
<td>9.308</td>
<td>-2.028</td>
<td>0.911</td>
<td>0.321</td>
</tr>
<tr>
<td>Ang-I + Quinaprilat</td>
<td>0.496</td>
<td>28.738</td>
<td>11.999</td>
<td>-1.437</td>
<td>0.946</td>
<td>0.387</td>
</tr>
<tr>
<td>Ang-I + Chymostatin</td>
<td>0.433</td>
<td>26.628</td>
<td>12.961</td>
<td>1.208</td>
<td>0.911</td>
<td>0.509</td>
</tr>
<tr>
<td>Ang-I + Losartan</td>
<td>0.264</td>
<td>29.547</td>
<td>9.158</td>
<td>1.052</td>
<td>0.952</td>
<td>0.334</td>
</tr>
<tr>
<td>Ang-II + Losartan</td>
<td>0.326</td>
<td>41.367</td>
<td>10.903</td>
<td>1.132</td>
<td>0.934</td>
<td>0.283</td>
</tr>
<tr>
<td>Ang-I + PD123,319</td>
<td>0.599</td>
<td>28.018</td>
<td>8.701</td>
<td>0.146</td>
<td>0.922</td>
<td>0.314</td>
</tr>
<tr>
<td>Ang-II + PD123,319</td>
<td>0.665</td>
<td>26.950</td>
<td>9.675</td>
<td>0.294</td>
<td>0.913</td>
<td>0.366</td>
</tr>
</tbody>
</table>

When the model fit was plotted with the experimental data, it can be seen that it does follow the shape of the stress strain curve (**Figure 5-5**). The model fit overlaps the experimental data which shows that by using the material constants in the Fung-type model the responses the aortic valve leaflets have to RAS can be modeled effectively.
Figure 5-5. The Fung-type constitutive fit along the experimental data in the circumferential and radial direction for the average response of each treatment.
Model Fit

The modified M-R isotropic model had a significantly lower $R^2$ than the modified M-R anisotropic model and the Fung-type model. This implies that it was the worst fit for the experimental data. The Fung-type model had the highest $R^2$.

Figure 5-6. The $R^2$ fit for each constitutive model in the 9 treatment groups

5.3.3 Fung-type material constants

The material constants of the Fung-type model were used to plot a constant strain energy contour over the strain field. This was done to verify the convexity of the strain energy function over a range of strains (75). Figure 5-7 shows that the plots for all the average treatment groups were all convex. This shows that the developed models are reliable. Contour plots were also done on each individual sample, and all samples satisfied the strain energy convexity constraints.
A non-linear anisotropy index (AI) can also be calculated using the material constants derived from the Fung-type model which is more ideal than the linear anisotropy index from aim 1. An isotropic response would have an AI value close to one, and the smaller the value of AI the more anisotropic the material is. The treatment groups had an average AI of 0.66 or lower implying that all treatment groups kept their anisotropic qualities. Control had the highest average AI at 0.664 for averaged samples and an AI of 0.637 for the average model. The AI was the lowest when treated with receptor antagonist with the AI being less than 0.34 for averaged individual samples in each treatment group. The AI was also low for the ang-I treated group and the ang-I supplemented with enzyme inhibitors. Ang-II had a lower AI than the control, but a higher AI than the ang-I treated group.
5.3.4 Anisotropy and cross-coupling

The average stress-strain behavior was used to identify the anisotropy and cross-coupling of the fibers (Figure 5-9). The anisotropy of a sample can be identified by the spacing between the circumferential and radial experimental data, and the cross-coupling of the fibers can be identified by analyzing the stress-strain behavior at the different loading ratios (addressed in aim 2). All treatment groups showed that the leaflets kept their anisotropic qualities as the spacing between the circumferential and radial loading directions is clear. However, the cross-coupling between the fibers was affected. In the control, a consistent spreading of the stress strain curves of the tension ratios can be seen in both the circumferential and radial direction. When treated with ang-I, the spreading decreases in the circumferential direction in comparison to the radial direction, and when treated with ang-II, the spreading decreases in the radial direction in comparison to the circumferential direction (Figure 5-9a,b). When ang-I was supplemented with an ACE inhibitor (quinaprilat), the spreading in the radial direction was slightly less than in the circumferential direction. Ang-I supplemented with a chymase inhibitor (chymostatin) had a consistent spreading in both the circumferential and radial direction, and had a higher spreading in the circumferential direction than the ang-I treated group (Figure 5-9a). When ang-I and ang-
II was supplemented with an AT-1R antagonist, it had a greater spacing between the circumferential and radial direction than the ang-I and ang-II treated groups implying that they were more anisotropic. The spreading of the stress strain curves in the circumferential and radial direction was also consistent in the ang-I supplemented with AT-1R antagonist, however when ang-II was supplemented with an AT-2R antagonist, the spreading was less in the circumferential direction. When ang-I and ang-II were supplemented with an AT-2R antagonist, they showed similar results to those supplemented with AT-1R antagonist.

**Figure 5-9.** 2nd P-K stress vs Green strain graph for tension ratios 1:1, 1:0.75, 0.75:1, 1:0.50, 0.50:1 for (A) enzyme and (B) receptor inhibitors of the average response based on identical
5.4 Discussion

Aortic valve tissue is an anisotropic material, and an anisotropic constitutive model was used to characterize the mechanical changes that occur due to RAS activation and inhibition. The modified M-R isotropic constitutive model was the worst fit model for the aortic valve tissue since it is a model used for isotropic materials. The $R^2$ ranged from 0.10 to 0.64 for individual samples and ranged from 0.27 to 0.44 in the average response model. The material constants were also not consistent between the samples in the same treatment group nor were they similar to the material constants derived from the average stress strain response model. The modified M-R anisotropic constitutive model was a better fit than the isotropic model with an $R^2$ ranging from 0.65 to 0.89 for each sample and an $R^2$ ranging from 0.78 and 0.89 for the average response for each treatment. However as it was derived from the Mooney-Rivlin model, the stress-strain curve was linear which was not consistent with the experimental data. The Fung-type model was the best fit model with an $R^2$ ranging from 0.75 to 0.98 for each individual sample. In the average model, the $R^2$ ranged from 0.91 to 0.95. When the model fit was graphed with the experimental data, it had the same stress strain response. The Fung-type model was the best constitutive fit to use to model the effects that RAS has on aortic valve tissue mechanics. The contour plots showed that the Fung-type models were all convex verifying that they do satisfy the strain energy convexity constraints. The AI calculated with the fung-type material constants showed that the samples maintained their anisotropic qualities. When samples were treated with receptor inhibitors, the AI was less than 0.5 implying that they were highly anisotropic. The stress-strain graphs for each treatment group showed a clear spacing between the circumferential and radial direction of loading, implying that the samples preserved their anisotropic qualities under all treatment groups. The treatment groups where ang-I and ang-II
were supplemented with an AT-1R had the greatest spacing in the stress-strain graphs and the lowest calculated AI. Though the control had the highest AI, it was seen that it had the greatest cross-coupling. This suggests that the stress in one direction affects the mechanical behavior in the orthogonal direction. This cross-coupling affect is important as the mechanical behaviors in the native valve are dependent of direction. In the next chapter, we will attempt to relate these results to changes in tissue biology.
CHAPTER 6 SPECIFIC AIM 3

Investigate the biological effects of mediators of the RAS system on aortic valves

6.1 Introduction

There are two phases during aortic valve disease progression: (I) the early initiation phase, and (II) the propagation phase. The initiation phase involves valvular lipid deposition, injury and inflammation (14). This phase has many similarities to atherosclerosis. The propagation phase is where pro-calcific and pro-osteogenic factors drive the progression of the disease (14). It is believed that the renin-angiotensin system (RAS) plays a role in the progression of aortic valve disease as the angiotensin-converting enzyme (ACE) is upregulated in calcified valves which facilitate angiotensin I (ang-I) to convert to angiotensin II (ang-II). This then affects the valve via the angiotensin II type I receptor (AT-1R) by mediation of its profibrotic effects. Angiotensin II type 2 receptor (AT-2R) mediation the antifibrotic and anti-inflammatory effects of ang-II, but differentiation of expression favors AT-1R (14).

The extracellular matrix (ECM) is composed of three different layers: the fibrosa, spongiosa, and ventricularis. The fibrosa is located near the aorta, the ventricularis near the left ventricle, and the spongiosa between both those layers (13). In a healthy valve, the fibrosa is predominately comprised of circumferentially-aligned type 1 collagen; the spongiosa is composed of proteoglycans; and the ventricularis is composed of a radially-aligned collagen/elastin network (30). Valve endothelial cells (VECs) are located in the outer layers of the leaflet, and within these layers, valve interstitial cells (VICs) are arranged in subpopulations. VECs maintain valve homeostasis, and the VICs secret ECM proteins to maintain valve strength and stability (16).
AS can proceed to calcific aortic valve disease (CAVD) from the initial cellular changes seen in the stenotic valve leaflets. Differentiation of VICs to other cell types, including myofibroblasts and osteoblasts, can be triggered from factors such as fluid shear stress, inflammatory cytokines, and growth factors (28). Inflammation in the valve is triggered by endothelial damage which release pro-inflammatory cytokines such as transforming growth factor-beta-1 (TGF-β1). This inflammatory activity activates the cells within the valve, and VICs differentiate from fibroblast-like cells to myofibroblast cells (5). As the disease progresses, VICs become further activated and differentiate into osteoblast-like bone-forming cells and respond to osteogenic mediators such as bone morphogenetic proteins (BMPs) which are member of the TGF-β superfamily (16). Calcified valves have an increased presence of TGF-β1 in the ECM and may contribute to the progression of CAVD by initiating apoptosis (6, 39). VECs may also undergo differentiation into osteoblast-like bone-forming cells through endothelial-to-mesenchymal-transformation (EnDoMT) in which they lose their endothelial cell properties. These transformed VECs begin to acquire phenotypic characteristics of myofibroblasts and express myofibroblast-specific markers such as α-SMA, Type I collagen, and vimentin. Similar to VICs, they can be stimulated by osteogenic mediators such as TGF-β and undergo osteogenic differentiation and fibrosis (16).

The increased production of collagen and the disorganization of the fibers are an indication of CAVD. In a diseased valve, the structure of the valve trilayer is disturbed. The crosslinking of the fibers is affected in the fibrosa layer while collagen production is affected in the other layers. In the fibrosa layer, fibers are shorter than that of those in a healthy valve, and in the spongiosa layer, the collagen fibers appear to become wider and denser. There are typically
more immature fibers in the spongiosa and are more mature fibers in the fibrosa during CAVD progression (30).

As phenotypic changes and alterations in tissue collagen are integral during the valve disease process, this aim focuses on investigating these biological effects that RAS has on the leaflet tissue. The phenotypic changes that VICs undergo due to RAS were investigated via immunohistochemistry, and the alterations in collagen fibers were investigated via quantitative polarized light microscopy and Picrosirius red staining.

6.2 Materials and Methods

Protocols can be found in Appendix A. MATLAB codes and instructions can be found in Appendix B.

6.2.1 Tissue treatment

Fresh porcine hearts (3-6 months old) were provided by Cockrum’s Custom Meat Processing and Taxidermy (Rudy, AR) or Braunschweig Processing (Neosho, MO), both FDA-approved abattoirs. Samples were transported in cold, sterile dPBS. The hearts were then dissected aseptically, and the left, right, and non-coronary aortic valve leaflets were pooled and washed in sterile dPBS. They were then supplemented with 150 U/ml penicillin/streptomycin in PBS for 10 mins.

The leaflets were then incubated in DMEM, 2% FBS, 150 U/ml P/S, and HEPES, and treated with the following. The binding of ang-I and ang-II to the receptors was investigated by inhibiting the AT-1R or AT-2R receptor subtypes. The samples were treated with either AT-1R inhibitor losartan (1μM) or AT-2R inhibitor PD123-319 (1μM) for 24 hours before being treated in combination with ang-I (10 μM) or ang-II (10 μM) for an additional 48 hours (69).
conversion of ang-I to ang-II was investigated by inhibiting ACE or chymase. Samples were treated with ACE inhibitor quinaprilat (1 μM) or chymase inhibitor chymostatin (10 μM) (69).

Following treatment, samples for immunohistochemistry and picrosirius red (PSR) staining were immediately placed in optimum cutting temperature (OCT) compound and flash frozen at -80°C. Tissues for quantitative polarized light microscopy (QPLI) were obtained from the biaxial testing experiments (Aims 1 and 2). The tissues were placed in a cryopreservation agent and flash frozen at -80°C.

**6.2.2 Immunohistochemistry**

Immunohistochemistry was performed against the primary antibodies rabbit anti- α-SMA (1:100, Abcam) and mouse anti-calponin (1:100, Abcam) in 2% goat serum and 1x PBS. Either 1:200 Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Life Technologies) was used to label primary antibodies of the proteins of interest. Secondary solution also contained 1:200 DAPI and 1x PBS.

Briefly, 5 μm thick sections were cryosectioned in the transverse plane and stored in -80°C freezer until staining. Slides were fixed in ice-cold acetone for 5 minutes and placed on a paper towel for 10 minutes to allow acetone to evaporate. A square was placed around each section with a water/alcohol proof marker and let dry for 10 minutes. Slides were rehydrated in 1x PBS for 5 minutes. Slides were placed in a humid chamber and 195 μL of 20% goat serum in 1x PBS was placed on each section as a blocking agent. They were incubated at 37 °C for 60 minutes. Blocking solution was removed by carefully holding the coverslip perpendicular to the bench and edge blotted with a kimwipe. 195 μL of desired primary antibody solution was placed on each section and incubated at room temperature for 2 hours in the humid chamber. Slides were washed by dipping three times in a staining jar containing 1x PBS and let sit for 5 minutes.
This was repeated three times in fresh 1x PBS. Slides were placed back in humid chamber and 195 µL of secondary antibody was placed on each sectioned. Samples were incubated for 2 hours at room temperature. Slides were washed again as stated before but in the dark to not photo-bleach the sample. Samples were mounted with pro-long gold and mounted media was allowed to cure for 24 hours. A thin layer of nail polish was placed around the cover slip and allowed to dry for 2 hours. Separate slides were stained with secondary antibody only and served as negative controls to assess non-specific binding of the secondary reagent. Slides were stored in the -20°C freezer until imaging. Images were acquired via a regular epifluorescence microscope.

**6.2.3 Picrosirius red (PSR) staining**

5 µm thick sections were cryosectioned in the transverse plane and stored in the -80°C freezer until staining. Slides were fixed in cold acetone for 5 minutes and then washed under running tap water for 10 minutes. Slides were then rinsed by dipping into distilled water three times. They were then placed on a paper towel, a few drops of phosphomolybdic acid 0.2% aqueous were placed on each tissue section, and treated for 5 minutes. Slides were placed in a glass staining jar containing 100ml of sirius red, 0.1% in saturated picric acid, for 90 minutes. Slides were washed for 2 minutes in 0.01N hydrochloric acid and then rinsed in 70% alcohol for 45 seconds. Slides were dehydrated by placing in 95% ethanol for 3 minutes two times and 100% ethanol for 3 minutes two times. They were cleared in xylene for 5 minutes two times and then mounted with cytoseal. Mounting medium was allowed to cure for at least 24 hours before imaging.

PSR stained samples were imaged via polarized light microscopy, and were subsequently analyzed using a custom MATLAB (Mathworks Inc.) script based on a published algorithm to quantify the red, orange, yellow, and green pixel count by their respective hue and saturation.
levels (68). The colors red, orange, yellow, and green correspond to the thickness of the collagen fibers present from thickest to thinnest respectively. The pixel count for each color was divided by the total pixel count for the four colors to acquire the percentage of each color present in the image.

6.2.4 Quantitative polarized light imaging (QPLI)

Tissue was in washed in dPBS after biaxial testing and placed in a solution of 85% DMEM, 5% FBS, and 10% dimethyl sulfoxide (DMSO) to prevent ice crystal from damaging the tissue when frozen, as a cryopreservation agent. Samples were flash frozen at -80°C. Prior to imaging, samples were defrosted by placing at room temperature for 30mins. A custom-built trans-illumination based QPLI microscope (Olympus BX51, Olympus Corp., Tokyo, Japan) setup was used which utilizes a rotating polarizer and circular analyzer (83, 84). Images were collected using a 20x objective (UPlanFL N 20x, 0.5 NA, Olympus Corp.) and data was collected using a 10x objective (UPlanFL N 10x, 0.3 NA, Olympus Corp.). Full field images were produced via image stitching. The images were analyzed using a custom MATLAB code. Average light retardation was measured as proportional to the amount of collagen in the tissue. A rotating polarizer and circular analyzer enabled the assessment of variance in collagen fiber alignment as a measure of collagen fiber orientation.

6.2.5 Statistical methods

Results are reported as mean with standard error. Data was first analyzed for normality using the Shapiro-Wilk normality test. For the enzyme inhibition, a one way ANOVA was run using multiple comparisons versus the diseased (ang-II) state using the Dunnett’s Method. For the receptor inhibition, a two way ANOVA was run using all pairwise multiple comparison
follow by the Tukey post hoc test for normally distributed data. Analyses were performed using Sigma Plot. A \( p \) value of less than 0.05 was used to indicate statistical significance.

6.3 Results

6.3.1 Cellular phenotypic changes in tissue

Via immunohistochemistry (IHC), we observed that activation or inhibition of the RAS pathway altered the valve cell phenotype in the valve tissue. \( \alpha \)-SMA and calponin were not expressed in the control samples (Figure 6-1a.I,II & b.I,II), implying that when treated with only media, the cells in the tissue were not being activated. When the tissue was treated with ang-I or ang-II, \( \alpha \)-SMA and calponin expression was upregulated, suggesting that the cells were being activated and becoming more contractile and myofibroblast-like (Figure 6-1a.III-VI & b.III-VI).

Additionally, the upregulation of these markers in the ang-I treated groups suggests that ang-I is being converted to ang-II locally on the valve leaflet. To validate that enzymes are present locally to convert ang-I to ang-II, valves leaflets were treated with an ACE and chymase inhibitors, quinaprilat and chymostatin respectively, prior to and during treatment of ang-I. The upregulation of \( \alpha \)-SMA and calponin seen when treated with ang-I alone was mitigated in the ACE and chymase inhibitor groups. This suggests that ang-I activates the cells due to it converting to ang-II locally through either ACE (Figure 6-1a.IX,X) or an ACE-independent pathway (Figure 6-1a.IX,X).

When the tissue was treated with an AT-1R inhibitor (losartan) in conjunction with ang-I or ang-II, \( \alpha \)-SMA and calponin were not expressed (Figure 6-1b.VII-X). This suggests that by inhibiting the ability for ang-II to bind to AT-1R the activation of the cells was being mitigated. When the tissue was treated with an AT-2R inhibitor (PD123,319) in conjunction with ang-I or ang-II, \( \alpha \)-SMA and calponin were expressed (Figure 6-1b.XI-XIV). This suggests that by
inhibiting the ability for ang-II to bind to AT-2R, ang-II is binding to AT-1R causing the activation of the cells. It should be noted that this last observation is supported in the literature as the AT-1R receptor subtype is known to be pro-atherosclerotic and AT-2R antagonizes its effects (55).

**Figure 6-1.** Representative micrographs of aortic valve leaflets immunohistochemically labeled with α-SMA (green), calponin (red) and DAPI (blue) treated with (a) enzyme inhibitors and (b) receptor inhibitors. Arrows indicate expressed areas. (scale bar=100µm)
6.3.2 Alterations of collagen structure

Next, we wanted to relate the mechanical observations from aims 1 and 2 to possible alterations in the collagen structure of the leaflets. To do so we utilized two techniques that leverage the birefringence of collagen fibers: picrosirius red staining (PSR) and quantitative polarized light imaging (QPLI).

In the enzyme inhibition treatment groups, ang-I (21.53±0.77) and ang-II (22.80±1.77) had a higher percentage of the thinnest fibers than the control (17.35±3.42). When ang-I was supplemented with the ACE (19.77±3.16) and chymase (20.32±2.57) inhibitors, they showed a higher percentage of thinnest fibers to the control but a lower percentage than the ang-I and ang-II treated groups (Figure 6-2b). The control (27.62±1.45), ang-I (28.24±1.52), and ang-II (28.63±0.97) treatment groups showed a similar percentage of thin intermediate fibers. The ang-I supplemented with ACE (25.76±2.55) and chymase (26.52±1.47) inhibitors showed a lower percentage of thin intermediate fibers (Figure 6-2c). The control (44.97±4.68) and ang-I supplemented with ACE (45.52±4.69) and chymase (46.84±4.02) inhibitors treatment groups had a similar percentage of thinnest and thin intermediate fiber percentage combined while the ang-I (49.77±0.91) and ang-II (51.43±2.51) treated samples showed a higher thinnest and thin intermediate fiber percentage combined (Figure 6-2a). The ang-I (36.43±0.60) and ang-II (37.18±2.01) treated group had a lower percentage of thick intermediate fibers than the control (40.29±1.80). The ang-I supplemented with ACE (38.18±2.02) and chymase (38.74±2.09) inhibitors had a lower percentage of thick intermediate fibers than the control but a higher percentage than the ang-I and ang-II treated groups (Figure 6-2d). The ang-I (13.81±1.47) and ang-II (11.39±1.15) treated group had a lower percentage of thickest fibers than the control (14.74±2.89). The ang-I supplemented with ACE (16.30±2.83) and chymase (15.70±2.96)
inhibitors had a higher percentage of thickest fibers than the control, ang-I and ang-II treated
groups (Figure 6-2e). The control (55.03±4.68) and ang-I supplemented with ACE (54.48±4.69)
and chymase (54.43±4.99) inhibitors treatment groups had a similar percentage of thickest and
thick intermediate fiber percentage combined while the ang-I (50.23±0.91) and ang-II
(48.57±2.51) treated samples showed a lower percentage of thickest and thick intermediate fiber
percentage combined (Figure 6-2a). This indicates that when samples are treated with ang-I and
ang-II there is more newly synthesized collagen fiber, and when the conversion of ang-I to ang-II
is inhibited by either ACE or an ACE-independent pathway, there is more mature fiber and
similar to what is seen when tissue is not treated with ang-I or ang-II.

Figure 6-2. (A) Quantification of picrosirius stain for enzyme inhibitors of collagen fiber
thicknesses of (B) thinnest, (C) thin intermediate, (D) thick intermediate, and (E) thickest
collagen fiber present. Red dotted line is the untreated, native leaflets.

In the receptor inhibition treatment groups, ang-II (28.88±4.62) had a higher percentage
of the thinnest fibers than the control (21.14±1.27) while ang-I (19.42±3.21) had a similar
percentage of thinnest fibers than the control. When ang-I was supplemented with AT-1R
antagonist (22.46±5.77), there was a higher percentage of thinnest fibers than the ang-I treated group, and when ang-II was supplemented with an AT-1R antagonist (19.94±5.26), it had a lower percentage of thinnest fibers and a similar percentage than the control. When ang-I was supplemented with an AT-2R antagonist (20.75±7.66), it had a similar percentage of thinnest fibers than the control and ang-I, but when ang-II was supplemented with AT-2R antagonist (25.87±4.81), it had a higher percentage of thinnest fibers than the control but lower than the ang-II treated group (Figure 6-3b). The control (34.07±1.57) had the highest percentage of thin intermediate fibers. The ang-I (26.83±.85) and ang-II (28.28±2.81) treated group had a lower percentage of thin intermediate fibers than the control. When ang-I was supplemented with an AT-1R antagonist (27.68±3.06), it had a similar percentage of thin intermediate fibers than the ang-I treated group. When ang-II was supplemented with AT-1R antagonist (25.57±3.69), it had a lower percentage of thin intermediate fibers than the ang-II treated group. When ang-I was supplemented with an AT-2R antagonist (26.57±2.8), it showed a similar percentage of thin intermediate fibers than the ang-I treated group. When ang-II was supplemented with AT-2R antagonist (26.84±2.34), it had a similar percentage of thin intermediate fibers than the ang-II treated group (Figure 6-3c). The ang-I (46.25±2.52) treatment group had a lower percentage of thinnest and thin intermediate percentage combined than the control (55.20±2.68). The ang-II (57.16±7.27) treatment group had a higher percentage of thinnest and thin intermediate percentage combined than the control and ang-I. The ang-I supplemented with AT-1R antagonist (50.14±8.69) treatment group had a similar percentage of thinnest and thin intermediate fiber percentage combined than the ang-I treatment group. The ang-II supplemented with AT-1R antagonist (45.51±8.94) treatment group had a lower percentage of thinnest and thin intermediate fiber percentage combined than the ang-II treatment group. The ang-I supplemented with AT-2R
antagonist (47.32±10.42) treatment group had a similar percentage of thinnest and thin intermediate fiber percentage combined than the ang-I treatment group and lower percentage than the control group. The ang-II supplemented with AT-2R antagonist (52.72±6.34) treatment group had a lower percentage of thinnest and thin intermediate fiber percentage combined than the control and ang-II treatment group (Figure 6-3a). Ang-II (33.04±3.41) had a lower percentage of the thinnest and thin intermediate fibers than the control (38.42±2.45) while ang-I (40.24±2.62) had a higher percentage of thick intermediate fibers than the control. When ang-I was supplemented with AT-1R antagonist (39.15±4.53), there was a similar percentage of thick intermediate fibers than the ang-I treated group, and when ang-II was supplemented with an AT-1R antagonist (40.72±4.92), it showed a higher percentage of thick intermediate fibers as the ang-II treated group. When ang-I was supplemented with an AT-2R antagonist (39.38±5.37), it had a similar percentage of thick intermediate fibers than the ang-I treated group, and when ang-II was supplemented with AT-2R antagonist (35.79±3.15), it had a higher percentage of thick intermediate fibers than the ang-II treated group but lower percentage than the control (Figure 6-3d). The control (6.38 ±0.76) had the lowest percentage of thick fibers. The ang-I (13.52±0.19) and ang-II (9.80±3.98) treated groups had a higher percentage of thick fibers than the control. When ang-I was supplemented with an AT-1R antagonist (10.71±4.29), it had a lower percentage of thick fibers than the ang-I treated group. When ang-II was supplemented with AT-1R antagonist (13.77±4.07), it had a higher percentage of thickest fibers than the ang-II treated group. When ang-I was supplemented with an AT-2R antagonist (13.29±5.06), it had a similar percentage of thickest fibers than the ang-I treated group and higher percentage than the control. When ang-II was supplemented with AT-2R antagonist (11.49±3.38), it had a higher percentage of thickest fiber percentage than the control and ang-II treated group (Figure 6-3d). The control
(44.80±2.68) and ang-II (42.84±7.27) treatment group had a similar percentage of thickest and thick intermediate percentage combined, and ang-I (53.75±2.52) and a higher percentage of thickest and thick intermediate than the control and ang-II treated group. When ang-I was supplemented with an AT-1R antagonist (49.86±8.69), it had higher thickest and thick intermediate percentage combined than the control and lower percentage to the ang-I treated group. When ang-II was supplemented with an AT-1R antagonist (54.49±8.94), it had a higher thickest and thick intermediate percentage combined than the ang-II and control. When ang-I was supplemented with an AT-2R antagonist (52.68±10.42), it had a similar thickest and thick intermediate percentage combined than the ang-I treated group and higher percentage than the control. When ang-II was supplemented with an AT-2R antagonist (47.28±6.34), it had higher thickest and thick intermediate percentage combined than the control and lower percentage than the ang-II treated group (Figure 6-3a).

Figure 6-3. (A) Quantification of picrosirius stain for receptor inhibitors of collagen fiber thicknesses of (B) thinnest, (C) thin intermediate, (D) thick intermediate, and (E) thickest collagen fiber present. Red dotted line is the untreated, native leaflets.
The QPLI set up uses a rotating polarizer and a circular analyzer allowing for the quantification of fiber amount and orientation via the measurement of average retardation (collagen amount) and the local directional variance (variance of the collagen orientation). The ang-I (35.83±4.26) treated group had a lower average retardation than the control (43.68±5.53) while the ang-II (47.55±3.17) treated group had a higher average retardation than the control. The ang-II treated group had the overall greatest average retardation (Figure 6-4a,b). When ang-I was supplemented with the ACE (40.98±4.95) and chymase (37.27±4.08) inhibitors, they showed a lower average retardation than the control and ang-II treated group but a higher average retardation than the ang-I treated group (Figure 6-4a). When ang-I was supplemented with an AT-1R antagonist (44.35±3.02), it had a higher average retardation than the ang-I treated group but similar average retardation than the control. When ang-II was supplemented with an AT-1R antagonist (41.72±3.06), it had a lower average retardation than the control and ang-II treated group. Ang-I supplemented with an AT-2R antagonist (28.20±4.43) had a lower average retardation than the control and ang-I treated group. Ang-I supplemented with an AT-2R antagonist (38.05±2.86) had a lower average retardation than the control and ang-II treated group (Figure 6-4b).

The ang-I (0.064+0.025) treated group had a lower higher local directional variance than the control (0.032±0.14) while the ang-II (0.022±0.007) treated group had a lower local directional variance than the control (Figure 6-4c,d). When ang-I was supplemented with an ACE (0.037±0.015) and chymase (0.052±0.017) inhibitors, it had a lower local directional variance than the ang-I treated group but higher local directional variance than the control and ang-II treated group (Figure 6-4c). When ang-I was supplemented with an AT-1R antagonist (0.035±0.020), it had a higher local directional variance than the control but lower variance than
the ang-I treated group. When ang-II was supplemented with an AT-1R antagonist (0.044±0.013), it had a higher local directional variance than the control and ang-II treated group. When ang-I was supplemented with an AT-2R antagonist (0.157±0.035), it had the highest local directional variance. Ang-II supplemented with an AT-2R antagonist (0.053±0.020) had a higher local directional variance than the control and ang-II treated group (Figure 6-4d).

**Figure 6-4.** Quantification of the average retardation (collagen amount) in the (A) enzyme and (B) receptor inhibitors and local directional variance (variance of collagen orientation) in the (C) enzyme and (D) receptor inhibitors using quantitative polarized light imaging. Red dotted line is the untreated, native leaflets.
6.4 Discussion

Immunohistochemistry (IHC) showed that RAS affects the phenotypic properties of the cells in the aortic valve tissues. Tissue treated with ang-I and ang-II showed an upregulation in expression of α-SMA and calponin implying that cells were becoming activated. When ang-I was supplemented with an ACE and chymase inhibitor, these effects were mitigated implying that by inhibiting the ability for ang-I to convert to ang-II cells maintain their quiescent state. This also strongly suggests that the effects seen by treating samples with ang-I is due to its ability to convert locally into ang-II. Ang-I and ang-II supplemented with an AT-1R receptor inhibitor also mitigated the effects of ang-I and ang-II mediated pathological signaling. This result indicates that the quiescent state can be maintained by inhibiting the binding of ang-II to AT-1R. When ang-I and ang-II were supplemented with an AT-2R antagonist, there was an upregulation of α-SMA and calponin. This indicates that when the ability for ang-II to bind to AT-2R is inhibited, ang-II will bind to AT-1R causing the cells to become activated.

The sample size for picrosirius red (PSR) staining was small (n=3) and so it was underpowered and did not show significant difference. However, PSR showed various trends, and the sample size will be increased to verify its significance. Tissue treated with ang-I and ang-II had a higher percentage of thin collagen fibers suggesting more newly synthesized fibers. This suggests that there is an increase in production of collagen which is a hallmark for early progression of valve fibrosis. When ang-I was supplemented with an ACE and chymase inhibitor, there was a similar percentage of thin fibers than that of the control suggesting that by inhibiting the ability of ang-I to convert to ang-II, the pathological increased production of collagen fiber is mitigated.
When ang-II was supplemented with an AT-1R antagonist, there was a lower percentage of thin fibers than the ang-II treated group, and when supplemented with an AT-2R antagonist, it had a similar percentage of thin fibers as the ang-II group. Overall, this suggested that by inhibiting that the binding of ang-II to AT-2R increases pathologic-like collagen production as seen in the ang-II groups alone.

In contrast to PSR, QPLI was conducted on tissue after biaxial mechanical testing. QPLI showed the maximum collagen amount in Ang-II, which matches PSR. Perhaps ang-II treatment increased collagen synthesis while reducing collagen degradation. AT-2R inhibitor seemed to reduce collagen amount significantly. QPLI showed that there was an increase in the variance of the collagen orientation in ang-I treated samples. When ang-I was supplemented with an ACE and chymase inhibitor, the collagen orientation was similar to that of the untreated leaflet. Ang-I and ang-II supplemented with an AT-2R antagonist also showed an increase in variance of collagen orientation while ang-I and ang-II supplemented with AT-1R had a similar directional variance than the control. This suggests that the binding of ang-II to AT-1R increases the disorganization of the fibers. By inhibiting the ability for ang-I to convert to ang-II and inhibiting the ability for ang-II to bind to AT-1R these effects are mitigated. There may be increased collagen degradation in the AT-2R inhibitor groups caused by elevated signaling at the AT-1R. The activation of VICs, an increase production of collagen fibers, and disorganization of collagen fibers are hallmarks of early disease progression. Two days of treatment were enough to alter phenotype, but not long enough to observe significant changes at the collagen fiber level. In the future, total collagen amount will be analyzed using a hydroxyproline assay.
CHAPTER 7 LIMITATIONS AND FUTURE DIRECTIONS

One of the limitations in the study was the amount of time the samples were treated. More time in culture was needed as there were very little changes in collagen observed. Though the effects could be seen when limiting ang-II to bind to just one receptor, the time given for ang-II to bind to either receptor when given the option to bind to both AT-1R and AT-2R was not enough. Though ang-II is prone to bind to AT-1R, a longer time period is needed to increase the potential binding to AT-1R over AT-2R. The study had to be limited to a 48 hour treatment of ang-I and ang-II as samples are more prone to become contaminated if treated for longer periods of time.

Another limitation was the mechanical testing in the current biaxial system. Our current loading cells are not capable of detecting very low tensions properly. The load cells in the upgraded biaxial system will allow for detection of the lower tensions seen in the toe region. This would allow for more sensitive reading of the stiffness in the toe region and also help in obtaining better constitutive model fits. In addition, our system was displacement based. This means that to stretch the samples to the desired tension multiple tests had to be run to determine the correct displacement needed for it. In addition, the samples were stretched close to their desired tension such as 90 N/m, but the tension could vary approximately from ± 7N/m on certain samples. This could affect the calculations using the max green strain such as anisotropy index and areal strain.

The alterations of the collagen measured using QPLI was done on samples after the biaxial testing. Using QPLI while running the mechanical tests would be ideal as it would provide information on the instantaneous alterations of the collagen fibers in real time. In addition, QPLI was done on a portion of the sample tested and not on the sample as a whole. The
current biaxial system did not have the ability to do this, but the biaxial system which we are currently upgrading will have the ability to do QPLI during the mechanical testing and image the sample as a whole and not just a small portion. In the future, the new biaxial system will eliminate these limitations seen during the mechanical testing and QPLI performed during this thesis.

In this study, we sought to investigate the affects that RAS has on aortic valve tissue mechanical and phenotypic properties. In the future, we will investigate how RAS affects the aortic valve at the cellular level. We conducted some preliminary studies on the effects that RAS has on valvular interstitial cells (VICs), but there were a few limitations in the study. Traditionally, VICs were grown in media containing basal Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). However, studies have shown that by growing VICs in this “classical DMEM” VICs become activated and exhibit myofibroblast qualities instead of the fibroblast-like qualities which quiescent VICs have (85). To overcome this, a study conducted by Latif, et al showed that growing VICs in DMEM supplemented with 2% FBS, 50 ng/ml insulin, and 10 ng/ml fibroblast growth factor-2 (FGF2) allow the cells to maintain their fibroblast phenotype (85). In our preliminary studies, we cultured VICs in this “fibroblast media” and treated them with ang-I, ang-II, ang-I supplemented with an AT-1R or AT-2R antagonist, and ang-II supplemented with an AT-1R or AT-2R antagonist in the same manner as we treated the tissue in our study. However, culturing cells in fibroblast media presented us with some limitations in the thin-film experiment and western blots. Thin-film experiments allow us to measure the contraction and relaxation of cells when stimulated with endothelin-1 (ET-1) and relaxed with HA-1077. In a previous study conducted by our lab, we were able to measure the contraction and relaxation of VICs treated with ang-II (68). In that
study, VICs had been cultured in classical DMEM. When we tried to conduct thin film experiments in VICs cultured in fibroblast media, the VICs were not strong enough to lift the thin film when they were stimulated. It the lifting and curling of the thin film that allows the measurement of the VICs contractility and relaxation. To overcome this limitation, we will be conducting traction force microscopy (TFM) to measure the mechanical properties of VICs instead of the thin film experiment. In TFM, VICs are cultured on an acrylamide membrane containing fluorescent beads. Using a multi-photon microscope, the beads can be tracked when the VICs are stimulated instead. The VICs in our preliminary study were also treated in a static state which means that the VICs were not undergoing strain as they are within their native environment in the body. When western blotting was done to determine the expression of vimentin and calponin, there was no significant difference between the expression of calponin or vimentin in the treatment groups (Figure 7-1). To overcome this limitation, VICs will be exposed to mechanical strain in all future studies.

Figure 7-1. (A) Western blot analysis of VICs treated with receptor inhibitors for vimentin and calponin. Expression of (B) vimentin and (C) calponin normalized with β-actin.
CHAPTER 8 SUMMARY AND CONCLUSIONS

The objective of this study was to investigate how renin-angiotensin signaling (RAS) pathway mediators affect the biological and mechanical function of the aortic valve tissue by further understanding the ACE or ACE independent pathway in which angiotensin I (ang-I) converts to angiotensin II and also the binding of ang-II to type 1 ang-II receptor (AT-1R) and type 2 ang-II receptor (AT-2R). This was done so by treating porcine aortic valve tissues with ang-I, ang-II, ang-I+ACE inhibitor (quinaprilat), ang-I+chymase inhibitor (chymostatin), ang-I+AT-1R antagonist (losartan), ang-II+AT-1R antagonist, ang-I+AT-2R antagonist (PD123,319), ang-II +AT-2R antagonist, or vehicle (DMEM).

In the first aim, aortic valve tissue was exposed to biaxial mechanical testing after being pre-treated. The samples were tested at five different tension load ratio protocols at a maximum tension of 90 N/m: \( T_{circ}:T_{rad} = 1:1, 1:0.75, 1:0.5, 0.75:1, 0.5:1 \). Key findings from this study are outlined below:

- The cross-coupling of the fibers was affected as can be seen by the spreading of the stress-strain curves in the 2\textsuperscript{nd} P-K stress vs Green strain graph
- The anisotropic properties were not affected as can be seen by the clear spacing between the circumferential and radial curves in the 2\textsuperscript{nd} P-K stress vs Green strain graph and a strain anisotropy index of less than 0.6.

In most cases, the mechanical properties exhibited by enzyme inhibitors were similar to the control which could suggest that when ang-I is not able to convert to ang-II it can sustain its native properties. However the mechanical properties exhibited by ang-I and ang-II were similar to the control as well. Though ang-II is more prone to bind to AT-1R, the amount of treatment time may not have been sufficient to increase the binding of AT-1R over AT-2R. However,
limiting the ability to bind to just one receptor did show a trend in mechanical behavior. In most cases, the tangent modulus was lower in samples treated with an AT-1R antagonist than those with an AT-2R antagonist implying that the binding of ang-II to AT-1R increases the stiffness of the sample. There was a high variation in the extensibility, and it seems to be highly unreliable because small changes in the high TM fit will result in wide changes in where the line cuts the x-axis, thereby affecting extensibility.

In specific aim 2, three phenomenological constitutive models were utilized to characterize the biomechanical changes that occur due to RAS mediators on aortic valves: (1) modified Mooney-Rivlin (M-R) isotropic model, (2) modified M-R anisotropic model, and (3) Fung-type model. Typically M-R constitutive models are used to characterize the behavior of isotropic materials, but the modified anisotropic model adds an anisotropic term to the modified M-R isotropic model. The Fung-type constitutive model is typically used to characterize the behavior of a non-linear anisotropic material. Material constants for each model was derived for each individual sample and averaged, and material constants were also calculated using data from an average stress strain response model. Key findings from this study are outlined below:

(1) The modified Mooney-Rivlin (M-R) isotropic model fit the experimental data the least having an R^2 ranging from 0.27 to 0.44 in the average response model for the treatment groups. It was the least adequate for the experimental data.

(2) The modified M-R anisotropic model fit the experimental data better than the isotropic model having an R^2 ranging from 0.78 and 0.89 for the average response for each treatment. However, it still had a linear-like stress-strain response for our strain range, making it inadequate for the experimental data.
(3) The Fung-type model had the best fit model with an $R^2$ ranging from 0.91 to 0.95 in the average response model for the treatment groups. The curve was also non-linear as seen in the experimental data.

The material constants derived from the Fung-type model can give us some insight on the convexity of the model and the anisotropy of the samples. The contour plots showed that the Fung-type models were all convex verifying that they do satisfy the strain energy convexity constraints. The treatment groups had an average AI of 0.66 or lower implying that all treatment groups kept their anisotropic qualities. Limiting the ability to bind to just one receptor did show a trend in the AI derived from the Fung-type model in which the AI was the lowest when treated with receptor antagonist implying that they are highly anisotropic. Samples treated with receptor inhibitors had the greatest spacing between curves and lowest AI implying that the samples became highly anisotropic.

It was clear that an anisotropic model would provide a better model fit to characterize the biomechanical changes that occur due to RAS mediators on aortic valves. Though the modified M-R anisotropic model was a good fit, a non-linear model such as the Fung-type model was the best phenomenological constitutive model.

In specific aim 3, we sought to understand the biological effects that mediators of the RAS system had on aortic valve tissues. The phenotypic changes the cells undergo on the tissue due to RAS were investigated via immunohistochemistry (IHC), and the changes that collagen fibers undergo were investigated with quantitative polarized light microscopy (QPLI) and picrosirius red staining (PSR).

VICs were becoming activated when aortic valve tissue was treated with ang-I, ang-II, and ang-I or ang-II supplemented with an AT-2R as an upregulation of $\alpha$-SMA and calponin was
seen. The upregulation in expression of α-SMA and calponin was mitigated in ang-I or ang-II supplemented with an AT-2R antagonist and ang-I supplemented with an ACE or chymase inhibitor implying that cells are maintaining their quiescent form.

PSR showed an increase in production of collagen fibers in tissue treated with ang-I and ang-II. When the binding of AT-1R was impeded, there was lower production of collagen fibers, and when the binding of AT-2R was impeded, there was higher production of collagen fibers. This suggests that the binding of ang-II to AT-1R increased the collagen production.

QPLI showed an increase in disorganization of collagen fibers. Ang-I and ang-II supplemented with an AT-2R antagonist also showed an increase in variance of collagen orientation while ang-I and ang-II supplemented with AT-1R had a similar directional variance than the control. This suggests that the binding of ang-II to AT-1R increases the disorganization of the fibers. QPLI showed the maximum collagen amount in Ang-II, which matches PSR. Perhaps ang-II treatment increased collagen synthesis while reducing collagen degradation.

This study showed that ang-I and ang-II activated VICs, increased production of collagen fibers, and increased disorganization of collagen fibers. By inhibiting the binding of ang-II to AT-2R and only to AT-1R, these same effects were shown. When ability for ang-I to convert to ang-II and inhibiting the ability for ang-II to bind to AT-1R was inhibited, these effects are mitigated.

In this study, we were able to see that ang-I had an effect on the aortic valve which leads to believe that it can be converted locally. Ang-II may increase in the plasma during illnesses such as hypertension (66), but ang-II production could also increase in the heart via ACE or chymase locally. We observed the activation valvular interstitial cells both in ang-I and ang-II treated tissue. The upregulation of α-SMA and calponin has been seen in calcified aortic valves.
(86), and we can infer that the activation we observed by RAS mediators can potentially lead to the progression of valve disease. Ang-II has also shown to increase the thickness and endothelial derangements in aortic valves which have been linked to early progression of aortic stenosis (87). Thickening and calcification in the aortic valve can lead to reduced opening of the valve and increased stiffness (13). In our study, we observed that RAS mediators altered the stiffness of the aortic valve in addition to other biomechanical behaviors observed in stenotic aortic valves. Quinaprilat and losartan showed to mitigate these effects at the whole-tissue level, and more studies need to be conducted to further understand if common ACE inhibitors and ARBs used to treat other diseases such as hypertension can also be used as therapeutic treatments for the prevention of the progression of aortic stenosis that could lead to more severe conditions such as calcific aortic valve disease (CAVD).

In conclusion, through this thesis we were able to see that RAS mediators affected the biological and mechanical functions of the aortic valve leaflet. The activation of VICS and increased production and disorganization of collagen fibers correlated to the stiffness of the tissue which is linked to aortic stenosis. The finite Fung-type constitutive model was the best fit model to characterize the changes in the mechanical properties of the tissue due to RAS mediators. Further understanding of the role of the local RAS pathway in potentiating early CAVD could lead to potential therapeutic treatments by targeting RAS mediators.
REFERENCES


APPENDIX A PROTOCOLS

Protocols listed here are for methods mentioned. Additional protocols for cell work not included in Chapter 4 but in preliminary studied are also included. MATLAB codes for analysis of methods are in Appendix B.
Preparing for Slaughterhouse trip to Collect Large Animal Samples

**Reagents and Materials:**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PBS</td>
<td>VWR #101076-194</td>
</tr>
<tr>
<td>70% EtOH</td>
<td>In spray-bottle</td>
</tr>
<tr>
<td>Penicillin-Streptomycin (10,000 U/mL)</td>
<td>ThermoFisher #15140122</td>
</tr>
<tr>
<td></td>
<td>(15 mL aliquots; -20°C freezer)</td>
</tr>
<tr>
<td>Distilled water</td>
<td>40 mL in 50 mL conical</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Materials</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 mL syringe</td>
<td>Sterile</td>
</tr>
<tr>
<td>Filter</td>
<td></td>
</tr>
<tr>
<td>6L container</td>
<td></td>
</tr>
<tr>
<td>Aluminum foil</td>
<td></td>
</tr>
<tr>
<td>Surgical tools</td>
<td>Large scissors, small scissors, scalpel handle, larger straight and smaller curved tweezers</td>
</tr>
<tr>
<td>Stainless steel tray</td>
<td></td>
</tr>
<tr>
<td>Absorbent pads (2)</td>
<td></td>
</tr>
<tr>
<td>250 ml beaker</td>
<td></td>
</tr>
</tbody>
</table>

**Protocol:**

*Calling slaughterhouses and schedules*

MSML works with two slaughterhouses. As of November 2018, the following is the contact information and schedules for them:

- **Cockrum’s Meat Processing (Rudy, AR)**
  - Phone number: (479) 474-3012
  - Receive pigs on Sundays and Thursdays. Slaughter on Mondays and Fridays.
  - Open Mon-Fri 8am-5pm; Saturday 8am-noon; Sunday 2pm-5pm
  - Call two days before as sometimes they slaughter a day early if they have to many

- **Braunschweig Processing (Neosho, MO)**
  - Phone number: (417) 451-3150
  - Receive pigs on Sundays, Tuesdays, and Wednesdays. Slaughter on Mondays, Wednesdays, and Thursdays.
  - Open Mon-Fri 8am-5pm; Saturday 9am-12pm

Always call day before close to closing time to see how many pigs they will have. Call the morning of to remind them to save the hearts and make sure what time the hearts will be ready. Time will vary as they slaughter beef prior to pork and time depends on how many beef they have.
Preparing diluted pen/strep (can be days before dissecting)
1. Thaw pen/strep in water bath
2. In hood, mix 1 mL pen-strep with 40 mL ddH\textsubscript{2}O
3. Sterilize with 30 mL syringe and filter
4. Aliquot in ~12 mL of reagent into three 15 mL conicals
5. Place in -20°C freezer

Preparing PBS (day before dissecting)
1. Prepare required amount of 1x PBS from 10x PBS stock. (Call slaughterhouse day before to know how many hearts will be available)
   a. 300 ml 10x PBS + 2700 ml ddH\textsubscript{2}O if picking up less than 3 hearts
   b. 200 ml 10x PBS + 1800 ml ddH\textsubscript{2}O if picking up more than 4 hearts. Max that can fill is 8
2. Fill appropriate sized polycarbonate containers with 1x PBS.
3. Cover top of container with aluminum foil.
4. Autoclave in liquid setting. Do not remove foil at any point when container is outside
5. Return autoclaved containers to biosafety hood in lab using sterile technique.
   a. Container is very hot after autoclaving. Either wait for it to cool down or use gloves to carry.
   b. Sterile technique: spray container with 70% alcohol before placing in hood
6. Remove foil, switch on UV light and expose overnight.

Preparing tools (day before dissecting)
1. Autoclave the following surgical tools in wrapped setting
   a. Small pack: Large scissors, small scissors, scalpel handle, larger straight and smaller curved tweezers
   b. Large pack: Stainless steel tray and absorbent pads (2 sheets)
   c. 250 ml beaker with top covered in aluminum foil
2. Place in lab bench until use
Biaxial stretching of soft tissues

**Important things to remember:**

- If using tissue samples, make sure it doesn’t dry out by constantly adding a few drops of deionized water.
- When you turn on the actuators sometimes one of the axis might make a noise trying to pull itself inside. Make sure to switch off the controller on the software and follow these steps for trouble shooting;
  - In the cycle menu, change the settings to regular mode.
  - Check all the actuators are being operated in position mode and not in load mode.
  - Bring all the actuators closer and change the settings back to biaxial mode in the cycle menu.
Reagents and Materials:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% Saline</td>
<td>Dissolve 54 grams of NaCl in 6 L of DI water</td>
</tr>
<tr>
<td>PDMS</td>
<td>For mold to fix sample</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Materials</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Sprayer</td>
<td>Keep sample moist at all times</td>
</tr>
<tr>
<td>Small and large petri dish</td>
<td>For PDMS mold and to cut samples</td>
</tr>
<tr>
<td>1cm x 1cm metal stamp</td>
<td>Used as stencil to cut sample</td>
</tr>
<tr>
<td>Cardboard frame</td>
<td>Sample is fixed to cardboard frame and is gripped onto grips</td>
</tr>
<tr>
<td>Staples</td>
<td>To fix sample to cardboard frame</td>
</tr>
<tr>
<td>Super glue</td>
<td>To administer tracking nodes</td>
</tr>
<tr>
<td>Black beads</td>
<td>To administer tracking nodes</td>
</tr>
<tr>
<td>Scissors</td>
<td>To cut cardboard frame</td>
</tr>
<tr>
<td>Razor blade and forceps</td>
<td>To excise sample and mount on frame</td>
</tr>
<tr>
<td>Water pump</td>
<td>To empty and fill water bath</td>
</tr>
</tbody>
</table>

Protocol:

Preparing material prior to day of testing

Prior to testing on day of

1. Place 6L of saline solution into water bath and turn on heating unit. Wait for at least 1 hour for solution to reach 37ºC.

Setting up system

1. Turn on all the actuators using the power knob under the desk.
2. Start the application named “MTL32” and once it loads, open “Testbuilder.”
3. In Testbuilder application, click on Proceed.
4. On the tab choose Test > Function generator.
5. On the right screen, open the tracking software named “Video Gauge.”
6. Transfer the 6 L of prepared saline into the bath and turn on the heater
7. Start the application Pylon Viewer
8. On the tab choose View>Devices and choose the Basler camera
9. On the tab choose View>Features and enable the acquisition frame rate and type 100 Hz for the frame rate
10. Mount the sample onto the cardboard frame and clamp them using grips.
11. Mark four points forming a square on the tissue sample using India ink and let it dry for a couple of minutes. (Will use 16 or 32 points later)
12. Mount the cardboard frame with the loaded sample onto one of the actuators and clamp it.
13. Now move the actuators one by one using the Jog function on the testbuilder screen and mount the other sides of the cardboard frame to the other actuators.

14. Cut the cardboard pieces in such a way that each actuator is gripping onto one side of the sample.

15. Use the DAQ settings to create a new file for each test and set the frame rate at 50 Hz.

16. Using the jog function bring all the actuators closer so that the sample is not expressing any load from any side.

17. Offset all the loads of the actuators. Setup > Offset readout.

18. Move each of the actuators until they all reach a tear load of 0.05N.

19. Offset all the positions of the actuators. Setup > Offset readout

20. Choose the Cycle function and choose General > Biaxial > input the dimensions of the samples. The Biaxial tab is where you can control if you want your actuators to work individually or biaxially. **Make sure to set it up at biaxial before running the test.**
21. Set waveform to “Ramp” with a mean strain of 0.75 and amp of 0.75. Ensure you select the “start point min” option to ensure the test begins at the relaxed state and proceeds to stretching. This will result in a displacement of 1.5 mm in each direction.
22. Run 20 cycles of preconditioning. Wait for 5 minutes.
23. Click Start on Pylon Viewer. Run and record three cycles of stretch. Click Stop on Pylon Viewer when cycles have been completed.
24. Once the test is done, click on “Report” and in the following screen choose single and click on “Single File”. This brings up another dialog box where you choose the file name that was created in the DAQ settings.
25. In the following screen, choose the file type as “CSV”; select all the loads for each actuator and export it. Save this CSV file to your desired location.

26. The saved file is ready to be analyzed using MATLAB.

27. Optional: For a quick view at the results, in the above shown screen you can choose “Preview” to look at the graphs.
### Immunohistochemistry

**Reagents and Materials:**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x PBS</td>
<td>100 ml 10x PBS</td>
</tr>
<tr>
<td></td>
<td>900 ml ddH2O</td>
</tr>
<tr>
<td>Acetone</td>
<td></td>
</tr>
<tr>
<td>Goat serum</td>
<td>-20ºC freezer</td>
</tr>
<tr>
<td>Primary antibodies</td>
<td>Look at primary antibody table</td>
</tr>
<tr>
<td>Secondary antibody</td>
<td>GAM 488 (green) (4 ºC fridge)</td>
</tr>
<tr>
<td></td>
<td>GAR 594 (red) (4 ºC fridge)</td>
</tr>
<tr>
<td></td>
<td>DAPI (blue) (-20ºC freezer)</td>
</tr>
<tr>
<td>Pro-long Gold</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Material</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastic slide jar and carrier</td>
<td></td>
</tr>
<tr>
<td>Water/Alcohol proof marker</td>
<td></td>
</tr>
<tr>
<td>Humid chamber</td>
<td></td>
</tr>
<tr>
<td>Coverslip</td>
<td></td>
</tr>
<tr>
<td>Slide box</td>
<td></td>
</tr>
<tr>
<td>Nail polish</td>
<td></td>
</tr>
</tbody>
</table>

**Protocol:**

**Preparing serum and antibodies**

For each section, make 200µL i.e. multiply number of sections by 200 and do calculations

Note: Following are with blocking serum as goat serum. Donkey serum can also be used, just replace goat serum with donkey serum and secondary as DAM 488 and DAR 594.

Example of calculations for 1 section:

1. 20% blocking serum
   a. 4 µL goat serum (20%)
   b. 196 µL PBS
2. Primary
   a. See table
3. Secondary
   Note: Keep in dark because it is fluorescent.
   a. 1 µL GAM 488 (green) (1/200)
   b. 1 µL GAR 594 (red) (1/200)
   c. 2 µL DAPI (blue) (1/100)
   d. 196 µL PBS
Fixation
1. Remove slides and allow to reach room temperature
2. Soak for 5 minutes in ice-cold acetone
3. Leave out for 5-10 mins till acetone evaporates
4. Put square sharpie mark around each section & wait 5-10 mins to dry
5. Put in a slide jar filled with 1x PBS to rehydrate section for 5 mins or more
   a. DO NOT TAKE OUT until ready to start block

Block
6. Place slides in humid chamber
   a. Place 1 slide at a time. Put blocking serum immediately as to not dry out the sample
7. Place 195 µl of 20% goat serum on each section
8. Incubate at 37°C for 30-60 mins

Primary Stain
9. Remove the slide from the blocker solution.
   a. DO NOT WASH!
10. Blot the slide. This is done by carefully holding the coverslip perpendicular to the lab bench. Once a bead of liquid builds up at the bottom of the coverslip, press the edge of the coverslip again a Kim Wipe to wick away the excess moisture
   a. Goal is NOT to completely dry the slide-simply remove excess PBS from glass surface.
11. For each section, place 195 µl of the primary.
12. Cover and incubate at room temperature for 1-2 hours in humid chamber

Wash 2
13. In plastic slide container, dip 3 x in 1x PBS and then let sit for 5 min
14. Repeat step 1 3x in new solution of PBS

Secondary
15. With a pipette place 195 µl of secondary antibody solution on each sections
16. Incubate for 1-2 hours in room temperature

Wash 3
17. Repeat wash 2 in dark area

Mount (in dark)
18. Place drop of Pro-long Gold ant fade reagent (brown bottle)
19. Cover slip and wait 12-24 hours
20. Then thin layer of nail polish around the cover slip
21. Let set for 2 hours
22. Once dry, they are ready for microscopy. When not imaging them, store in a slide box or a dark box in 20C freezer. Slides will keep for several months but deteriorate over time.
## Primary & Secondary Antibodies

<table>
<thead>
<tr>
<th>Primary</th>
<th>Species</th>
<th>Block</th>
<th>Concentration</th>
<th>Primary for 1 slide</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1 (-20 freezer)</td>
<td>Rb</td>
<td>Goat</td>
<td>1/25</td>
<td>8 µl TGF-β1 4 µl (2%) Goat 188 µl PBS</td>
<td>Goat anti-rabbit GAR 594</td>
</tr>
<tr>
<td>RUNX2 (-20 freezer)</td>
<td>Ms</td>
<td>Goat</td>
<td>1/50</td>
<td>4 µl RUNX2 4 µl (2%) Goat 192 µl PBS</td>
<td>Goat anti-mouse GAM 488</td>
</tr>
<tr>
<td>Ki-67 (-20 freezer)</td>
<td>Rb</td>
<td>Goat</td>
<td>1/250</td>
<td>0.8 µl TGF-β1 4 µl (2%) Goat 195.2 µl PBS</td>
<td>Goat anti-rabbit GAR 594</td>
</tr>
<tr>
<td>Vimentin (-20 freezer)</td>
<td>Ms</td>
<td>Goat</td>
<td>1/300</td>
<td>0.67 µl vimentin 4 µl (2%) Goat 195.33 µl PBS</td>
<td>Goat anti-mouse GAM 488</td>
</tr>
<tr>
<td>AT-1 (-20 freezer)</td>
<td>Rb</td>
<td>Goat</td>
<td>1/100</td>
<td>2 µl AT-1 4 µl (2%) Goat 194 µl PBS</td>
<td>Goat anti-rabbit GAR 594</td>
</tr>
<tr>
<td>α-SMA (-20 freezer)</td>
<td>Ms</td>
<td>Goat</td>
<td>1/200</td>
<td>1 µl α-SMA 4 µl (2%) Goat 195 µl PBS</td>
<td>Goat anti-mouse GAM 488</td>
</tr>
<tr>
<td>Calponin (-20 freezer)</td>
<td>Rb</td>
<td>Goat</td>
<td>1/100</td>
<td>2 µl Calponin 4 µl (2%) Goat 194 µl PBS</td>
<td>Goat anti-rabbit GAR 594</td>
</tr>
</tbody>
</table>

### Secondary Antibodies that we have

| GAR 594 (red) (Goat-anti rabbit) | GAM 488 (green) (Goat-anti mouse) |
Picrosirius Red Protocol

Reagents:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphomolybdic Acid 0.2% Aqueous</td>
<td></td>
</tr>
<tr>
<td>Sirius Red, 0.1% in Sat'd Picric Acid</td>
<td></td>
</tr>
<tr>
<td>0.01 N Hydrochloric Acid</td>
<td></td>
</tr>
<tr>
<td>70% Alcohol</td>
<td></td>
</tr>
<tr>
<td>Xylene</td>
<td></td>
</tr>
<tr>
<td>95% Ethanol, 100% Ethanol</td>
<td></td>
</tr>
<tr>
<td>Ultrapure water (ddH$_2$O)</td>
<td></td>
</tr>
<tr>
<td>Cytoseal mounting medium</td>
<td>Electron Microscopy Sciences #18006</td>
</tr>
<tr>
<td>Coplin/staining jars</td>
<td>Electron Microscopy Sciences #71388-01</td>
</tr>
</tbody>
</table>

Protocol:

1. Let slides come close to room temperature
2. Fix in cold acetone for 5 min.
3. Wash in running tap water, 10 minutes.
4. Rinse in distilled water.
5. Treat in Phosphomolybdic Acid 0.2% Aqueous (1-5 minutes). Place the slides on a paper towel and put a few drops of the acid on each tissue section.
6. Stain in Sirius Red, 0.1% in Sat'd Picric Acid, for 90 minutes. Note: You can filter and re-use sirius red stain.
7. Wash for 2 minutes in 0.01 N Hydrochloric Acid.
8. Rinse in 70% Alcohol for 45 seconds.
9. Dehydrate:
   a. EtOH 95% (3 min, 2x)
   b. EtOH 100% (3 min, 2x)
10. Clear in xylene (5 min, 2x).
11. Mount with cytoseal in fume hood and coverslip.
12. Let mounting medium cure for 24 h before imaging.

Results:
Collagen Fibers
Order of colors from least mature to most mature (thinnest fibers to thickest fibers):
Green < Yellow < Orange < Red
Protein Isolation

Reagents and Materials:

<table>
<thead>
<tr>
<th>Reagent/Material</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIPA Lysis Buffer System</td>
<td>Santa Cruz # sc-24948</td>
</tr>
<tr>
<td></td>
<td>1x lysis buffer (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>PMSF in DMSO (200mM)</td>
</tr>
<tr>
<td></td>
<td>Protease inhibitor cocktail (PIC) in DMSO</td>
</tr>
<tr>
<td></td>
<td>Sodium orthovanadate (NaVO) in water (100mM)</td>
</tr>
<tr>
<td>1xPBS</td>
<td></td>
</tr>
<tr>
<td>0.5 and 1.5 mL microcentrifuge tube</td>
<td></td>
</tr>
</tbody>
</table>

Protocol:

1. Culture cells or tissues under the appropriate conditions (if relevant). Proteins are typically extracted using lysis buffers. We use a strongly denaturing urea lysis buffer as outlined below. You may wish to use other lysis buffer formulations if you don’t want the protein to be as denatured or if you are running the gel in native conditions.

2. For every 1mL of lysis buffer, add 10μL of PMSF, PIC, and NaVO.

3. For cells:
   a. Wash cells 3x with sterile dPBS.
   b. Aspirate all PBS
   c. Add ~ 30-60μL of cold lysis buffer per coverslip and scrape using cell scraper.
   d. Using a 100μL pipet, suction up and down until the solution becomes less viscous (~10 times).
   e. Transfer to a clean 0.5mL microcentrifuge tube.

4. For tissues:
   a. Wash tissue 3x with sterile dPBS.
   b. Pulverize tissue in a mortar and pestle under liquid nitrogen.
   c. Transfer pulverized tissue to a clean microcentrifuge tube.
   d. Add cold lysis buffer. In general, we use 3mL of lysis buffer per gram of tissue. (I.e. for a 5x5 mm valve leaflet sample, we would use 200μL lysis buffer).

5. Centrifuge at max speed, 4°C, for 5 mins to pellet cell/tissue debris. 0.5mL tubes too small for the centrifuge so put inside capless, empty 1.5 mL tube or tube will break.

6. Carefully withdraw supernatant into a separate clean 1.5 mL microcentrifuge tube labeled with sample information.

7. Quantify protein yield using the micro-BCA protein assay kit.
BCA Protein Assay

Reagents and Materials:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIPA Lysis Buffer System</td>
<td>Santa Cruz # sc-24948</td>
</tr>
<tr>
<td></td>
<td>1x lysis buffer (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>PMSF in DMSO (200mM)</td>
</tr>
<tr>
<td></td>
<td>Protease inhibitor cocktail (PIC) in DMSO</td>
</tr>
<tr>
<td></td>
<td>Sodium orthovanadate (NaVO) in water (100mM)</td>
</tr>
<tr>
<td>Micro BCA Protein Assay Kit</td>
<td>ThermoFisher # 23235</td>
</tr>
<tr>
<td></td>
<td>Micro BCA Reagent A, B, &amp; C</td>
</tr>
<tr>
<td></td>
<td>Albumin Standard Ampules (BSA), 2mg/mL</td>
</tr>
</tbody>
</table>

Protocol:

Preparing Samples

1. Label microcentrifuge tubes with BCA, lysis, and the names of each of your samples. You need one tube per sample.
2. Add the appropriate amount of ddH₂O water to each microcentrifuge tube, depending on what dilution you want to use:
   a. For 1:100: Add 45 μL of ddH₂O
   b. For 1:200: Add 47.5 μL of ddH₂O
3. Add 5 μL (for 1:100) or 2.5 μL (for 1:200) of each sample to appropriate microcentrifuge tube. Mix by vortexing.
4. Make up the dilute BSA solution (final - 0.4mg/ml)
   a. 320 μL ddH₂O
   b. 80 μL 2 mg/mL BSA (vial in the kit)
5. Make up the dilute lysis buffer solution (or whatever solvent your proteins are in):
   a. Stock: For every 1mL of lysis buffer, add 10μL of PMSF, PIC, and NaVO.
   b. Dilution: 360 μL ddH₂O + 40 μL stock lysis buffer
### Adding in 96 well plate

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>C0</td>
<td>C0</td>
<td>S1</td>
<td>S1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>C4</td>
<td>C4</td>
<td>S2</td>
<td>S2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>C8</td>
<td>C8</td>
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Do duplicates for each control and sample well

6. For each control well, add the following amount of ddH₂O:
   a. C0 → 90 µL
   b. C4 → 89 µL
   c. C8 → 88 µL
   d. C16 → 86 µL
   e. C24 → 84 µL
   f. C32 → 82 µL
   g. C40 → 80 µL

7. Add 90 µL of MilliQ water to each Sample well.
8. Add 10 µL of the dilute lysis buffer to each control well.
9. Add the following dilute BSA to each control well:
   a. C0 → 0 µL
   b. C4 → 1 µL
   c. C8 → 2 µL
   d. C16 → 4 µL
   e. C24 → 6 µL
   f. C32 → 8 µL
   g. C40 → 10 µL

10. Add 10 µL the dilute sample to the appropriate well in the 96-well plate.
11. Calculate the amount of BCA reagent that you need: 100 µL / well
12. Make up the BCA Assay reagent (Pierce kit)
    a. Add 50% Solution A
    b. Add 48% Solution B
    c. Add 2% Solution C
    d. Mix
13. Add 100 µL of the reagent to each well
14. Incubate at 37°C for 30-60 min (you need to optimize this)  
15. Read the absorbance at 562 nm on the plate reader.

**Calculating protein concentration**  
16. Average the absorbance reading for the duplicates  
17. Subtract the absorbance of CO from the absorbance of all samples to normalize  
18. Graph the normalized absorbance of the control wells vs known concentration of control wells and calculate the equation of the best fit line  
   a. \( y = \text{absorbance} \) and \( x = \text{known concentration} \) (0,4,16, etc.)  
19. Using the equation, calculate the concentrations of sample with their absorbance  
20. Samples were diluted by 100 so multiply the calculated concentration with 100 to calculate true concentration
**Gel Electrophoresis**

**Protocol:**

*Sample Preparation*

To get a good signal in the gel scanner, approximately 5-20μg of protein is recommended per well of an electrophoresis gel. Prepare your gel samples in a 0.5 mL microcentrifuge. Sample for SDS-Page consists of the protein sample + 4x sample buffer + reducing agent (β MeEth) + ddH₂O. Amount of total sample depends on gel and the volume each well can take.

1. Divide amount of protein (i.e. 10 μg) by the concentration of the specimen sample and multiply by 1000 to calculate the amount of sample volume (μL) needed.
2. ¼ of volume is the sample buffer with or without the reducing agent.
   a. Sample buffer with reducing reagent is 90% 4x sample buffer and 10% β MeEth.
   b. Sample without reducing reagent is used only for the standard.
3. Add the volume of the protein sample, 2x sample buffer, and reducing agent and subtract by the total volume needed to calculate the volume of ddH₂O.
4. Mix sample
   a. Tip: It’s easiest to put the water first into the 0.5 mL microcentrifuge tube, then the reducing agent, and last the specimen volume. Can label the 0.5 mL microcentrifuge tube as well as pipetting in the amount of water days before running the gel. For best results, wait till the morning to pipette in the reducing agent and specimen sample.
5. Boil sample at 95°C for 5 min using heating block. This ensures complete denaturation and breakage of any disulfide links in the protein.
6. Condensation will happen so spin the samples down and the water will go back to the bottom of the tube.

*Standard Preparation*

The standard is what shows the molecular weight bands on the membrane. You can have more than 1 lane of standard depending on how many samples you have and as long as there is at least one lane in front of your set of samples, i.e. if you have 16 samples and an 18 well gel, you can put one in the 1st and 18th well, if you have 14 samples, you can put one in the 2nd and 17th well, or if you have 17 samples, you can only put in the 1st well. Standard consists of the protein standard + 4x sample buffer + ddH₂O.

1. Multiply the number of lanes by the volume per well to get total volume.
2. Multiply 5 by the number of lanes for amount of protein standard.
3. Divide total volume by 4 for amount of 4x sample buffer.
4. Subtract amount of protein standard and 4x sample buffer from the total volume for amount of ddH₂O.
5. Mix sample. DO NOT BOIL!
   a. Tip: It’s easiest to put the water first into the 0.5 mL microcentrifuge tube, then the reducing agent, and last the protein standard.
Running the Gel Electrophoresis

1. Prepare the appropriate amount of buffer for the electrophoresis depending on the type of gel you are running:
   a. Tris-HCl/TGX gel: Tris/glycine/SDS running buffer
      i. 100 ml buffer + 900 ml ddH2O
   b. Tris-acetate gel: Tricine buffer
   c. Bis-tris gel: MES or MOPS buffer

2. Each precast gel is packaged individually in a plastic storage tray, which can also be used as its own staining dish.

3. Remove tape from bottom of the cassette.

4. Insert precast gels in electrophoresis tank making sure that the integral buffer chamber faces the center of the tank.

5. Fill the integrated upper buffer chamber with running buffer (~60mL).

6. Carefully remove the comb and top up any spilt running buffer. DO NOT THROW THE COMB AWAY AT THIS POINT!

7. Fill each half of the lower buffer tank with ~400mL running buffer to the marked fill line.

8. Load samples using a pipet with gel loading tips. Using the sample loading guide will ensure that there are no “parallax” errors.

9. Load protein standard to appropriate lane (s).

10. Place lid on the tank, aligning the color coded banana plugs with the corresponding jacks on the lid. Run gels as follows. Run time is approximately 45 – 65 min.
   a. Tris-HCl: 200V constant
   b. TGX: 200V constant
   c. Tris-acetate: 150V constant
   d. Bis-tris: 200V constant

11. After electrophoresis is complete, turn off power supply and disconnect the electrical leads.

12. Remove the lid from tank and remove gel and discard running buffer.

13. Insert comb into the slits on either side of the cassette and gently crack open the cassette.

14. Pull the two halves of the cassette apart to completely expose the gel. BE CAREFUL! THE GEL IS EXTREMELY FRAGILE!

15. If you are going to do a western blot, proceed to that protocol. For staining the gel go to the next section.
Western Blotting

Protocol:

Before starting:

- Make sure you know what antibodies you will be doing as the molecular weight of the primaries dictates where the membranes will be cut. Can do 2 antibodies on the same membrane with similar MW if different species (rabbit and mouse). Otherwise, cut the membrane so as to have thinner strips.
- Have at least 1.5L cold transfer buffer already made.

Preparation for Transfer/Western blot

1. When the electrophoresis is halfway done, cut a section of PVDF membrane of approximately the same size as the gel. DO NOT TOUCH THE MEMBRANE EVEN WITH GLOVED HANDS AS THAT WILL CAUSE TRANSFER OF “SELF” PROTEINS WHICH WILL CONFOUND YOUR RESULTS!
2. The PVDF membrane is extremely hydrophobic so we need to “prewet” it. Dip it in methanol, and then soak it in cold Transfer buffer on the belly button shaker in the 4°C fridge for the remainder of the duration of the electrophoresis. You can use the tray that came with the gel for this purpose.
3. Recipe for 10L 1x transfer buffer is (scale for your needs accordingly) as follows. Store buffer in the 4°C fridge.
   i. 2L methanol
   ii. 1L 10x TG buffer
   iii. 7L ddH₂O
4. Prepare sponges by adding transfer buffer on the plate and soak them. Roll out all bubbles. Leave in transfer buffer and re-roll right before assembly incase bubbles were formed.
5. After electrophoresis is complete, turn off power supply and disconnect the electrical leads.
6. Remove the lid from tank and remove gel
   a. Save the running buffer as it can be reused at most 3 times.
7. Insert comb into the slits on either side of the cassette and gently crack open the cassette.
8. Pull the two halves of the cassette apart to completely expose the gel. BE CAREFUL! THE GEL IS EXTREMELY FRAGILE!

Western blot

1. In the provided plastic tray, assemble the Bio-rad transfer cassette as follows, starting from black side (negative) to red (positive):
   a. Sponge
   b. Filter paper
   c. Gel
   d. PVDF membrane
   e. Filter paper
f. Sponge

2. When assembling the above, make sure you don’t rub or smudge the gel with the PVDF membrane. The PVDF membrane is extremely protein-philic, so any smudge of the gel (which is full of your protein samples) will appear when you are scanning the membrane.

3. It is absolutely crucial that the PVDF membrane is on the red (positive) terminal. If you reverse the gel and PVDF membrane, the proteins will travel the opposite way and will go out of the cassette into solution. That is, you have lost everything!

4. Once the assembly has been made, use the roller to roll out any air bubbles as presence of bubbles will appear as empty circles on your PVDF membrane scan.

5. Close and lock cassette and assemble in Bio-Rad transfer tank. **Don’t forget to insert cooling block as tank gets very hot when running.**

6. Run western/transfer blots in the 4°C fridge under the following conditions:
   a. Standard (1 hour): 100V constant voltage
   b. Overnight (16 hours): 10V constant voltage

7. At the end of the transfer step, disassemble cassette and place PVDF membrane protein side up on gel cassette and using the molecular weight markers as a guide, cut PVDF membrane horizontally into regions of interest. Can use the comb as a guide but make sure to clean it first with distilled water. **YOU MUST USE A CLEAN RAZOR BLADE AND COMB!**

8. Immediately place membrane strips in full strength Li-cor blocking buffer. Use the black Li-cor boxes for this purpose.

**Immunolabeling**

This procedure is similar to immunostaining, in the sense that it involves blocking, primary and secondary antibody incubations.

**Blocking**

1. Blocking is done in full strength Li-cor blocking buffer for 1.5 hours at room temperature on belly dancer (speed 0.5).
   a. 10 ml for long membrane and 5 ml for small membrane (can vary as long as covered)

2. When done, store blocking buffer in a 100-250ml container and store in 4°C. Can be reused at most 3 times.

**Primary antibody incubation**

3. Prepare appropriate dilution of primary antibody in full strength Li-cor buffer.

4. Place a 300-500μL drop of antibody on petri dish (can have multiple membranes but have to be same primaries) and invert membrane onto the drop. Amount depends on length of membrane. This is similar in technique to immunostaining of coverslips.

5. Incubate the membranes in primary antibody for 16 hours (overnight) at 4°C.

**Washing**

6. Make washing buffer. One quarter strength Li-cor blocking buffer diluted in PBS (1:3).

7. Return the membranes face up, and wash in washing buffer 3 times, 5 min each on the belly dancer (speed 4).

8. During this time, pipette the primary from the petri dish into a 1.5ml Eppendorf and store in -20°C. Can be reused at most 3 times.
Secondary antibody incubation (*LIGHT-SENSITIVE*)

9. Prepare appropriate dilution of secondary antibody during washing (cover conicals with aluminum).
   a. 1/15,000 DAM 700 or DAR 800
   b. 1:1 dilution of Licor buffer in PBS
   c. 15 ml for long membranes and 10 ml for small membranes. Can vary as long as covered but more than washing as it is highly important that it is completely immersed since speed is slower on belly dancer and incubation is longer.

10. Incubate membranes in secondary antibody for 1 hours at room temperature on belly dancer (speed 1).
    a. If done in black Licor box, it can just be placed on belly dancer, but if done in a box that allows light to go through, cover them with a cardboard box that won’t allow light in (i.e. careful with holes on box).

**Washing**

11. Discard the secondary and wash the membranes in washing buffer 3 times, 5 min each on the belly dancer.
12. Wash the membranes in ddH$_2$O 2 times, 5 min each on the belly dancer.

**Imaging**

1. Clean odyssey scanner using ddH$_2$O and Kim wipes only.
2. Place membranes face down on the glass and orient them appropriately.
3. Cover the membranes with the thick rubber mat and use the roller to roll out air bubbles. This roller is larger and different from the one you used for the western/transfer blot step.
4. Close the odyssey scanner lid.
5. Open the Odyssey program and do: File \(\rightarrow\) New
6. Select your folder and click open.
   a. Password: Admin
7. On toolbar click blue icon with blue arrow
8. Another window will open. With mouse double click on grid that is shown, and drag the box to match where your membranes were. Click flip image
    a. The greater the area, the longer the scan
    b. The greater the resolution, the slower the scan
9. Select the channel in which you want to scan your membrane. For antibodies labeled with IR680, select channel 700. For antibodies labeled with IR800, select channel 800. Both channels can be selected at once, but the scan will be slower.
10. Select intensity. The starting point is usually “5”.
11. Select appropriate resolution, and thickness setting.
12. Click scan. Once scan runs, you can save your image and use the Odyssey software to analyze your bands.

**Tips:**
If you want stronger signal, you can place the membranes on the scanner, and let them completely dry up (protected from light) before placing the rubber mat etc.
APPENDIX B MATLAB CODE

MATLAB codes listed here are to analyze methods mentioned in methods sections of each chapter. Each code has instructions on how to use the codes.
Biaxial Testing MATLAB Instructions

**Use:**
Manual to analyze data acquired from biaxial mechanical testing.

**Images:**
Collected during biaxial testing from Basler AC640 camera. 100 images of desired stretch.

**Tension:**
Collected during biaxial testing from biaxial mechanical tester (Test resources) and software provided by test resources.

**Measurements:**
Thickness of sample measured by placing sample in OCT compound after biaxial testing, sectioning the samples with the cryotome, imaging with darkfield microscope, and measuring length with imageJ.

**MATLAB Instructions:**

MATLAB codes required to analyze data: Biaxial_1.m, Biaxial_2.m, Biaxial_3.m, Biaxial_4.m, automarkertrack.m, leastsq.m.

**Troubleshooting:**

In earlier testing, tensions were being read accurately in all directions. However at a later time point, x1 is not able to read the tension being applied in the x1 direction properly. The code must be changed accordingly to average or not average the tensions in the x direction. There may be times where there can also be an issue in the y direction. Check and change the following lines in the code if you desire to use only one directional tension or the average tension of both.

**Biaxial_1.m**

Line 102:
For average x, \( x_{tension}(j) = (x1x2(\text{row}(j))) - (x1x2(\text{row}(j)-49)) \);
For only x1, \( x_{tension}(j) = (x1(\text{row}(j))) - (x1(\text{row}(j)-49)) \);
For only x2, \( x_{tension}(j) = (x2(\text{row}(j))) - (x2(\text{row}(j)-49)) \);

Line 103:
For average y, \( y_{tension}(j) = (y1y2(\text{row}(j))) - (y1y2(\text{row}(j)-49)) \);
For only y1, \( tension(j) = (y1(\text{row}(j))) - (y1(\text{row}(j)-49)) \);
For only y2, \( y_{tension}(j) = (y2(\text{row}(j))) - (y2(\text{row}(j)-49)) \);
Biaxial_2.m
No changes

Biaxial_3.m
Line 116:
For average x, plot ([1:50],x1x2s);
For only x1, plot ([1:50],x1s);
For only x2, plot ([1:50],x2s);

Line 119:
For average y, plot ([1:50],y1y2s);
For only y1, plot ([1:50],y1s);
For only y2, plot ([1:50],y2s);

Biaxial_4.m
Line 29:
For average x, x_ten=data([1:50],5);
For only x1, x_ten=data([1:50],1);
For only x2, x_ten=data([1:50],2);

Line 30:
For average y, y_ten=data([1:50],6);
For only y1, y_ten=data([1:50],3);
For only y2, y_ten=data([1:50],4);
Biaxial_1.m

This code creates a matrix containing the max x and y tension to allow the user to choose the stretch that was stretched to the most accurate tension. This is due to the fact that our biaxial machine is not load bearing but by displacement so the tension can vary through test. In addition, our biaxial machine does not detect low tensions well so even though it may look like the tension reached the desired tension, once subtracted by the starting tension, it may be different. When testing user does choose a stretch that looks good, but this code allows the user to make sure that it is the best.

Input:
Biaxial Machine csv file: x1,x2,y1,y2

Output:
Matrix: x_tension and y_tension on command window (in order of stretches)

Instructions on how to use this in following biaxial codes:

This code will allow the user to determine which stretch has most appropriate tension. Make sure to write down the stretch for further use. User analyzes the deformation of that stretch in biaxial_2.m and calculates the needed tensions in biaxial_3.m.

Notes:
File must be .csv, have no text, and at least the following columns:
Column B (2nd) is Rel. LOAD - X1 (N)
Column C (3rd) is Rel. LOAD - Y1 (N)
Column D (4th) is Rel. LOAD - X2 (N)
Column E (5th) is Rel. LOAD - Y2 (N)

Instructions:

1. Delete all words in .csv file.
2. Drag file to MATLAB folder
3. Run code
4. Input how many stretches were done
5. Choose stretch that best fits ratio
6. In excel sheet, put how many stretches were done, what stretch will be analyzed, and what the tensions are of that stretch
Biaxial_2.m

This code is used to analyze the deformation the sample undergoes during testing. Samples must have 4 marks on the surface (2x2 square array) to trace and must be consecutive. Code will trace the movement of the markers during testing from the 100 images provided.

**Input:**
- Images collected from Basler AC640 camera
- MATLAB functions: automarkertrack.m and leastsq.m

**Output:**
- Two figures will pop up: figure (1) plot of Exx and (2) plot of Eyy. This is to insure that the code tracked the points correctly and did not skew. If they are not a peak, repeat analysis and choose a different area in the point that could be tracked easily.

![Figures](image)

- Excel file: deformresults.xlsx.
  - File contains Exx, Eyy, Exy, Ezz, E1, E2, dir1, dir1, dir2, and dir2. This file will be used in Biaxial_3.m.
  - Will be saved in output folder
- Two MATLAB data files: defg.mat and lamz.mat.
  - These will be used in Biaxial_4.mat.
  - Will be saved in output folder

**Note:**
- The function leastsq.m is used in the function automarkertrack.m.
- If there is noise when tracking the dots, change the pixel and num in the function automarkertrack.m
  - Make sure to write down the ideal pixel and num on excel sheet for future use.

**Instructions:**
1. Change path to folder containing the images. (Will always change)
a. Click the drop down menu and copy and paste the path name that appears onto line 27 of the code (path1= “pathname”).
   Ex. path1= ‘J:\Biaxial\20171230\ AngII \test 18_1_0.5\stretch 2+’;

2. Change file name to retrieve images (Will always change)
   a. Click on first image of the set of images you will be analyzing and copy and paste name onto line 28 of code (name1= “filename”).
   b. Erase the last three numbers of file name
      Ex. File name: “Basler acA640-750um (21856823)_20171230_185607817_0389” to “Basler acA640-750um (21856823)_20171230_185607817_0”
      name1= 'Basler acA640-750um (21856823)_20171230_185607817_0';

3. Change output file path (Will be same as long as in same computer path)
   a. Click the drop down menu and copy and paste the path name that appears onto line 29 of the code (path1= “outputpathname”).
   b. Make sure to add \point at the end
      Ex. out1= 'C:\Users\jxp040\Desktop\test\point';
      Note: Make output folder same folder that contains the MATLAB codes

4. Run program
5. User will be asked to input the number of the first image on command window
   Ex. 389
6. Click on 4 points to track in the following order:
   3---------4
   /         /
   /         /
   2---------1
   Note: Click on an area in the point that is in the center and dark
Biaxial_3.m

This code creates an excel sheet containing x1,x2,y1,y2 and average of x1&x2 and y1&y2 from the csv file provided by the biaxial machine containing the forces from different axis for the first 50 points as well as the Exx and Eyy for the first 50 points.

Input:
Excel file: deformresults.xlsx

Output:
- Four plots: x axis load, y axis load, Exx and Eyy.

- Excel file will be saved in output folder: rawdata.xlsx. File contains x1, x2, y1, y2, x1x2, y1y2, Exx, and Eyy. These will be used in Biaxial_3.mat.

Instructions:
1. Run program
2. Choose csv file provided by the biaxial testing machine
   a. Words should have been deleted prior to running biaxial_1.m and placed in folder
   b. The columns must contain the following information:
      A: Time
      B: Rel. LOAD - X1 N
      C: Rel. LOAD - Y1 N
      D: Rel. LOAD – X2 N
      E: Rel. LOAD – Y2 N
3. Input how many stretches were done
4. Input what stretch will be analyzed
Biaxial_4.m

This code creates an excel sheet E11, E22, S11, S22, P11, and P22 by using the excel sheet rawdata.xlsx from biaxial_3.m and mat files defg and lamz from biaxial_2.m. The excel file plotdata.xlsx can then be used to graph stress and strain data on sigma plot.

Input:
- Excel file: rawdata.xlsx
  - x1,x2,y1,y2,x1x2, y1y2, Exx, and Eyy
- Mat files: defg.mat and lamz.mat
  - defg.mat: deformation gradient
  - lamz.mat: lambda
- Measurement: sample thickness

Output:
- Excel file: plotdata.xlsx.
  - Will be saved in output folder.
  - File contains E11, E22, S11, S22, P11, and P22.
  - These will be used in sigma plot to create graphs and MATLAB to make constitutive models.

Instructions:
1. Run program
2. Input the thickness of the samples in mm
   a. Note: width is assumed to be 1cm.
Final Instructions

Cut and paste all output files into sample folder.
- Csv file from biaxial machine
- Excel files: deformresults.xlsx, rawdata.xlsx, and plotdata.xlsx
- MATLAB files: defg.m and lamz.m
- Txt files: point1.txt, point2.txt, point3.txt, and point4.txt

Graphing and statistical analysis
- Use the information on plotdata.xlsx
- Data can be grouped by condition or ratios used for that particular sample. Discuss with PI.
Biaxial Testing MATLAB Codes

To calculate the stress and strain for each tested sample, four MATLAB codes and two MATLAB functions were used. Biaxial_1 allows the user to choose which stretch had the most adequate force applied as our biaxial machine is displacement based. Biaxial_2 tracks the four-dot array to calculate the deformation gradient and strain. Biaxial_3 extracts the force and displacement data for the chosen stretch (50 data points as it was at 100 Hz). Biaxial_4 calculates the stress and output the stress and strain. The automarkertrack function automatically tracks the 4-dot array and the pixel size can be altered depending on the size of the dot being tracked. If the camera was move to where the x axis was in the y axis, the images need to be rotated and Rotate.m MATLAB code can be used to do so.
Biaxial_1.m MATLAB Code

% Biaxial Part 1

close all
clear all
clc

% Written by Jessica Perez
% Version June 2018

% University of Arkansas
% Department of Biomedical Engineering
% Mechanobiology and Soft Materials Laboratory (MSML)
% PI: Dr. Kartik Balachandran

% This code creates a matrix containing the max x and y tension to allow the
% user to choose the stretch that was stretched to the most accurate
% tension. This is due to the fact that our biaxial machine is not load
% bearing but by displacement so the tension can vary through test. In
% addition, our biaxial machine does not detect low tensions well so even
% though it may look like the tension reached the desired tension, once
% subtracted by the starting tension, it may be different. When testing
% user does choose a stretch that looks good, but this code allows the user
% to make sure that it is the best.

% Input:
% Csv file: x1, x2, y1, y2

% Output:
% Matrix: x_tension and y_tension on command window (in order of stretch)

% Instructions on how to use this in following biaxial codes:
% See which stretch has most appropriate load and use images of that
% stretch in biaxial part 2 and input the stretch number in biaxial part 3.

% Notes:
% File must be .csv, have no text, and at least the following columns:
% Column B (2nd) is Rel. LOAD - X1 (N)
% Column C (3rd) is Rel. LOAD - Y1 (N)
% Column D (4th) is Rel. LOAD - X2 (N)
% Column E (5th) is Rel. LOAD - Y2 (N)

% choosing file
[filename, pathname] = uigetfile('*.csv', 'Select file');
data = csvread(filename);
index = size(data,1);

% Note: currently x1 doesn't read properly so we only look at x2. See
% instructions on what lines to change if all tensions are reading properly.

% creating matrix of x1,x2,y1,y2 load from file data
i=1:index;
x1=data([1:index],2);
x2=data([1:index],4);
y1=data([1:index],3);
y2=data([1:index],5);
x1x2=mean([x1,x2],2);
y1y2=mean([y1,y2],2);

%plot tension. This allows user to see how many stretches were done and %what tensions may not be reading properly
figure(1)
subplot(2,3,1);
plot ([1:index],x1 );
title ('x1 load');
subplot(2,3,2);
plot ([1:index],x2 );
title ('x2 load');
subplot(2,3,3);
plot ([1:index],x1x2 );
title ('x1x2 load');
subplot(2,3,4);
plot ([1:index],y1 );
title ('y1 load');
subplot(2,3,5);
plot ([1:index],y2 );
title ('y2 load');
subplot(2,3,6);
plot ([1:index],y1y2 );
title ('y1y2 load');

%prompt user to how many stretches there were.
prompt='How many stretches were done? ';
st=input(prompt);

%Each stretch has a peak when graphed. This find the peaks that correspond %to each stretch
pks = findpeaks(x2);
max = sort( pks, 'descend' );
n = max(1:st);

%index the row for the max of each stretch
for j=1:st;
    q=n(j);
    r(j)=find(x2==q);
end

%row where max is for desired stretch
row=(sort(r, 'ascend'));

%difference between forces of peaks at DAQ of 100 Hz and frequency of 1 Hz. %i.e 100 data points, peak at 50 data points, subtract 49 to zero out
for j=1:st;
x_load(j)=(x2(row(j)))-(x2(row(j)-49)); %only use x2 and not x1
y_load(j)=(y1y2(row(j)))-(y1y2(row(j)-49)); %use y1y2
end

y_load
x_load
Biaxial_2.m MATLAB Code

Contact Dr. Kartik Balachandran for code.
%Biaxial Part 3

close all
clear all
clc

%Written by Jessica Perez
%Version June 2018

%University of Arkansas
%Department of Biomedical Engineering
%Mechanobiology and Soft Materials Laboratory (MSML)
%PI: Dr. Kartik Balachandran

%This code creates an excel sheet containing x1,x2,y1,y2 and average of
%x1&x2 and y1&y2 from the csv file provided by the biaxial machine
%containing the forces from different axis.

%Input:
% CSV file: Forces from biaxial Machine
% Excel file: derform deformresults.xlsx

%Output:
% Excel files: rawdata.xlsx
% Four plots: x axis load, y axis load, Exx and Eyy.

%Notes:
% File must be .csv, have no text, and at least the following columns:
% Column B is Rel. LOAD - X1 (N)
% Column C is Rel. LOAD - Y1 (N)
% Column D is Rel. LOAD - X2 (N)
% Column E is Rel. LOAD - Y2 (N)

%Instructions:
% 1. Run program
% 2. Choose csv. file
% 3. Input how many stretches were done
% 4. Input what stretch will be analyzed

%choosing file
[filename,pathname]=uigetfile('*.*.csv', 'Select file');
data=csvread(filename);
index=size(data,1);

%creating matrix of x1,x2,y1,y2 from file data
i=1:index;
x1=data([1:index],2);
x2=data([1:index],4);
y1=data([1:index],3);
y2=data([1:index],5);
x1x2=mean([x1,x2],2);
y1y2=mean([y1,y2],2);
% Prompt how many stretches were done
prompt='How many stretches were done? '; st=input(prompt);

% Prompt what stretch will be analyzed
prompt='Which stretch will be analyzed? '; sn=input(prompt);

% Each stretch has a peak when graphed. This find the peaks that correspond
% to each stretch
pks = findpeaks(x2);
max = sort( pks, 'descend' );
n = max(1:st);

% Index the row for the max of each stretch
for j=1:st;
    q=n(j);
    r(j)=find(x2==q);
end

% Row where max is for desired stretch
row=sort(r, 'ascend');
snrow=row(sn);

% Index first and last value of stretch (1-50)
f=snrow-49;
l=snrow;

% First 50 values for x1, x2, y1, and y2 of desired stretch
x1s=x1([f:l])-x1(f);
x2s=x2([f:l])-x2(f);
y1s=y1([f:l])-y1(f);
y2s=y2([f:l])-y2(f);
x1x2s=x1x2([f:l])-x1x2(f);
y1y2s=y1y2([f:l])-y1y2(f);

% First 50 values of E11 and E22 from deformresults.xlsx output from
% Biaxial_2.m
def=xlsread ('deformresults.xlsx');
E11=def([1:50],1);
E22=def([1:50],2);

% Output file
raw=[x1s,x2s,y1s,y2s,x1x2s,y1y2s,E11,E22];
h=['x1', 'x2', 'y1', 'y2', 'x1x2', 'y1y2', 'E11', 'E22'];
m=[h;num2cell(raw)];
filename='rawdata.xlsx';
xlswrite(filename,m);

% Output figure of first 50 E11, E22, and average x and y load
figure(1)
subplot (2,2,1)
plot ([1:50], E11);
title ('E11');
subplot (2,2,2)
plot ([1:50], E22);
title ('E22');
subplot (2,2,3)
plot ([1:50], x2s); % xl load is not reading properly so x value is just x2s
title ('x load');
subplot (2,2,4)
plot ([1:50], y1y2s);
title ('y load');
Biaxial_4.m MATLAB Code

%Biaxial Part 4

close all
clear all
clc

%Edited by Jessica Perez
%Version June 2018
%Written by Prashanth Ravishankar, 2016

%University of Arkansas
%Department of Biomedical Engineering
%Mechanobiology and Soft Materials Laboratory (MSML)
%PI: Dr. Kartik Balachandran

%This code creates an excel sheet E11,E22,S11,S22,P11,and P22 by using
%the excel sheet rawdata.xlsx from biaxial_3.m and mat files defg and lamz
%from biaxial_2.m. The excel file plotdata.xlsx can then be used to graph
%stress and strain data on sigma plot.

%Input:
% Excel file:rawdata.xlsx
% mat files: defg.mat and lamz.mat

%Output:
% Excel file: plotdata.xlsx

data=xlsread('rawdata.xlsx');
x=data([1:50],2); %x value is just x2 because x1 load was not reading
y=data([1:50],6); %y value is y1y2
E11=data([1:50],7);
E22=data([1:50],8);

%Assume width is 1cm and thickness 1mm so area equals 10^-5
prompt= 'What is the thickness of the sample (mm)? ';
t=input(prompt)*10^-3; %in mm
w= 1*10^-2; %assumed 1 cm
A=t*w;

%1st P-K (P) equals tension/area
P11=x./A./1000; % (Unit kPa)
P22=y./A./1000; % (Unit kPa)

%2nd P-K (S) equals F^-1 x P
load('defg.mat'); %gives you F [ lam1 0; 0 lam2]
load('lamz.mat'); %gives you lam3 i.e F33

F11=F(1:50,1);
F22=F(101:150,2);

F11_i=F11.^(-1); %inverse of F
F22_i=F22.^(-1); % inverse of F

S11=F11_i.*P11; % (Unit kPa)
S22=F22_i.*P22; % (Unit kPa)

% Cauchy stress (sigma) equals (1/J)x(F x P)

J=1 % J is the measure of volume change, assume no volume change so J=1
sigma11=(1./J).*F11.*P11; % (Unit kPa)
sigma22=(1./J).*F22.*P22; % (Unit kPa)

%Areal strain and anisotropy index
AS=E11.*E22; % areal strain is Ecirc*Erad
ASmax=max(AS)
AI=max(E11)./max(E22) % anisotropy index is Ecirc/Erad

output=[E11, E22, S11, S22, P11, P22, AS];
h={'E11', 'E22', 'S11{KPa}', 'S22{KPa}', 'P11{KPa}', 'P22{KPa}', 'AS'};
m=[h;num2cell(output)];
filename='plotdata.xlsx';
xlswrite(filename,m);

lam1=F([1:50],1);
lam2=F([101:150],2);
lam3=lamZ([1:50],1);

output=[lam1, lam2, lam3, sigma11, sigma22];
h={'lam1', 'lam2', 'lam3', 'sigma11', 'sigma22'};
m=[h;num2cell(output)];
filename='LambdaandCauchystress.xlsx';
xlswrite(filename,m);
%Automarker function used in Biaxial_2.m

%Written by Kartik Balachandran

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%PI: Dr. Kartik Balachandran

%Instructions:
% Change pixel and num accordingly to avoid noise

function N=automarkertrack(M,N)

pixel=50;
num=10;

distance=[-pixel:pixel]';

count=1;
for angle=0:pi/num:pi/num*(num-1)
    p=[distance*cos(angle)+N(1), distance*sin(angle)+N(2)];
    p=round(p);
    [s1 s2]=size(p);
    int=[];
    for i=1:s1
        int(i)=M(p(i,2),p(i,1));
    end

    fitpol=leastsq(distance,int',8);
    int2=fitpol{1};
    beta=fitpol{2};
    beta=flipud(beta);
    s1=length(beta);
    index=[1:s1-1]';
    p=beta(1:s1-1).*index;
    r=roots(p);

%this portion has been changed due to difficulty in tracking
    a=find(abs(r)==min(abs(r)));
    if length(a)>1
        a=a(1);
    end
    centroid(count)=real(r(a));
    count=count+1;
end

angle=[0:pi/num:pi/num*(num-1)]';


cosine=cos(angle);
sine=sin(angle);

centroid2=[centroid' .* cosine, centroid' .* sine];
[s1 s2]=size(centroid2);
centroid3=sum(centroid2)/s1;
N=N+centroid3;
Leastsq.m MATLAB Function Code

%Leastsq function used in function automarkertrack to be used in Biaxial.m

%Written by Kartik Balachandran

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%Mechanobiology and Soft Materials Laboratory (MSML)
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%least square fit to find Gfit.
%z is unvaried x-axis parameter
%ord is the order of the polynomial used for the fit

%Basic principles:
% fitted values \( \{G_{fit}\}=X\{beta\} \)
% \{beta\} is vector of coefficients
% For least square, \([X]\{beta\}-\{G\}\) is orthogonal to \([X]\)
% thus \([X]'([X]\{beta\}-\{G\})=0\)

function results=leastsq(z,G,ord)

zz=ones(length(z),1);
X=[zz];

for i=1:ord
    zz=z.*zz;
    X=[X zz];
end

beta=inv(X'*X)*X'*G;
Gfit=X*beta;
results={Gfit beta};
Rotate.m MATLAB Code

clc
clear all
close all

%Paths and file names
path1='C:\Biaxials Fresh\analyzed images\Ang-II+Los_FB4b\test 19_0.5_1\stretch 3'; %where original images are
path2='C:\Biaxials Fresh\analyzed images\Ang-II+Los_FB4b_rotate\test 19_0.5_1\stretch 3'; %where rotated images go
name1='Basler acA640-750um (21856823)_20180830_191536482_0'; %image name without last three numbers

%Frame number
prompt='What is the number of the first image? ';
f=input(prompt);

%Last image
l=f+99;
ind=[f:l];
[s1 s2]=size(ind);

pathnfile1=[path1 '\' name1 sprintf('%3.3d',ind(1)) '.tiff'];
M1=imread(pathnfile1,'tiff');
M1=imrotate(M1,90);
M2=imshow(M1);
BaseName=name1;
FileName=[path2 '\' BaseName,num2str(f),'.tiff'];
imwrite(M1,FileName,'tiff');

for j=1:99;
    pathnfile1=[path1 '\' name1 sprintf('%3.3d',ind(1+j)) '.tiff'];
    M1=imread(pathnfile1,'tiff');
    M1=imrotate(M1,90);
    M2=imshow(M1);
    BaseName=name1;
    FileName=[path2 '\' BaseName,num2str(f+j),'.tiff'];
    imwrite(M1,FileName,'tiff');
end
Average Model MATLAB Instructions

Prior to running the MATLAB code for the average model, an excel sheet containing the average x and y force provided by the system the E11 and E22 calculated by MATLAB code. The information can be found in the rawdata.xls file exported from MATLAB code Biaxial_3. Note: the x1 load cell was not functional so the x2 force was used as the average x force.

For example: If the treatment group is control, all control tests for ratio 1:1 must be in one excel sheet. The columns would be as followed. First sample: column A: x average force; column B: y average force; column C: E11; column D: E22; column E: empty. Repeat for every sample into next column i.e. second sample: column F: x average force; etc.

In addition, another excel file containing the thickness of each sample in column A must be made. For example: If there were 3 samples for the control at 1:1 force, A1 would be the thickness of the first sample, A2 the second sample, and A3 the third sample. The thicknesses must correspond to the information on the previous excel sheet. For example: the thickness in A1 must correspond to the information for that sample in the in columns A-D of the previous excel sheet. The thickness in A2 must correspond to the information for that sample in the in columns F-I of the previous excel sheet. The thickness in A3 must correspond to the information for that sample in the in columns K-N of the previous excel sheet.
%Average Model

cclc
clear all
close all

%%Written by Jessica Perez
%%Version January 2019

%University of Arkansas
%Department of Biomedical Engineering
%Mechanobiology and Soft Materials Laboratory (MSML)
%PI: Dr. Kartik Balachandran

%This code calculates the average stress and strain of n samples for one
%tension ratio.

%Equation:
% Pavg=Tavg/n * sum(1/h)
% Favg=sqrt(2*Eavg+1)
% Savg=Pavg*Favg^(-T)

%Input:
% Excel files:
% (1)File containing tension and strain data for n# samples for one
tension ratio (see notes (1) for order of information)
% (2)File containing thicknesses (mm) of each sample being averaged in
% column A in order

%Output:
% Command window:
% (1) max areal strain and (2) anisotropy index
% Excel files:
% (1) AveragePlotData.xlsx
% Contains: Average Green Strain, 2nd P-K stress, 1st P-K stress and
% areal strain for circumferential and radial direction
% (2) AverageEandTandStandardError.xlsx
% Contains: Average Green strain and tension in circumferential and
% radial direction and the standard error
% Graphs that need to be saved as jpeg or other filetype:
% (1) Average 2nd P-K stress vs average Green strain of circumferential
% and radial direction with error bars
% (2) Average 2nd P-K stress vs average Green strain of circumferential
% and radial direction without error bars
% Legend:
% Circumferential=Black
% Radial=Red

%Notes:
% (1) To extract the files the data should be as follows for however many
% sample numbers there are being averaged of one tension ratio:
% n1: Column A=x2; Column B=y1y2; Column C:Exx; Column D:Eyy
% n2: Column F=x2; Column G=y1y2; Column H:Exx; Column I:Eyy
% n3: Column K=x2; Column L=y1y2; Column M:Exx; Column N:Eyy

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% etc ..... 
% (2) Figures have no titles, x and y labels, or legends. I put those when 
% I'm compiling figures in a powerpoint. However, the code to do so is 
% commented so you can just uncomment them for whatever you want on 
% the graph.

% Extracting all control thickness from excel file and indexing 
disp('Select thickness file ')
[filename,pathname]=uigetfile('*.*', 'Select file');
t=xlsread(filename);
n=size(t,1);

% Extracting all control data from excel file and indexing 
disp('Select data file ')
[filename,pathname]=uigetfile('*.*', 'Select file');
data=xlsread(filename);
index=size(data,1);

% Creating matrices for the average x and y force and green strain (Exx and 
% Eyy) according to number of samples (n) 
for j=1:n
    col_x(j)=l+(5.*(j-1));
    x(j)=[data([1:50],col_x)];
    col_y(j)=2+(5.*(j-1));
    y(j)=[data([1:50],col_y)];
    col_Exx(j)=3+(5.*(j-1));
    E11(j)=[data([1:50],col_Exx)];
    col_Eyy(j)=4+(5.*(j-1));
    E22(j)=[data([1:50],col_Eyy)];
end
x=cell2mat(x(n));
y=cell2mat(y(n));
E11=cell2mat(E11(n));
E22=cell2mat(E22(n));

% x and y tension 
w=10.^-2; % (m) assume width 1 cm 
Txx=x./w; % (N/m) force/width 
Tyy=y./w; % (N/m) force/width

% Average tension (Tavg,ii) and standard deviation 
Txx_avg=mean(Txx,2);
Txx_sem=std(Txx,0,2)./sqrt(n);

Tyy_avg=mean(Tyy,2);
Tyy_sem=std(Tyy,0,2)./sqrt(n);

% Average green strain (Eave,ii) 
E11_avg=mean(E11,2);
E11_sem=std(E11,0,2)./sqrt(n);

E22_avg=mean(E22,2);
E22_sem=std(E22,0,2)./sqrt(n);

% Average P
% Pavg,ii=Tavg,ii/n * Sum(1/h)

t=t.*(10.^-3); %(m) h in mm and need in m
P11_avg=Txx_avg./n.*(sum(1./t));
P11_avg=P11_avg./1000; %kPa
P22_avg=Tyy_avg./n.*(sum(1./t));
P22_avg=P22_avg./1000; %kPa

%Average F
% Favg,ii=sqrt(2*Eavg,ii+1)
F11_avg=sqrt(2.*E11_avg+1);
F22_avg=sqrt(2.*E22_avg+1);

%Average S
% Savg,ii=Pavg,ii*Favg,ii^(-T)
F11_avg_i=1./(F11_avg);
F22_avg_i=1./(F22_avg);
S11_avg=(F11_avg_i.*P11_avg);
S22_avg=(F22_avg_i.*P22_avg);

%Average areal strain and anisotropy index
ASavg=E11_avg.*E22_avg; %areal strain is Ecirc*Erad
ASavg_max=max(ASavg)
AIavg=max(E11_avg./E22_avg) %anisotropy index is Ecirc/Erad

output=[E11_avg, E22_avg, S11_avg, S22_avg, P11_avg, P22_avg,ASavg];
h={'E11 avg', 'E22 avg', 'S11 avg', 'S22 avg', 'P11 avg', 'P22 avg','AS avg'};
m=[h;num2cell(output)];
filename='AveragePlotData.xlsx';
xlswrite(filename,m);

output=[E11_avg,E11_sem, E22_avg, E22_sem, Txx_avg, Txx_sem, Tyy_avg,
Tyy_sem];
h={'E11 avg', 'E11 std', 'E22 avg', 'E22 std', 'Txx avg', 'Txx std', 'Tyy
avg', 'Tyy std'};
m=[h;num2cell(output)];
filename='AverageEandTandStandardError.xlsx';
xlswrite(filename,m);

%Figures

%Standard error graph
E11_sem=std(E11,0,2)./sqrt(n);
S11_std_spot=S11_avg(4:2:end);
S11_std_spot_th=transpose([S11_std_spot S11_std_spot S11_std_spot]);
E11_std_spot=E11_avg(4:2:end);
E11_std_bar=E11_sem(4:2:end);
E11_std_pos = E11_std_spot + E11_std_bar;
E11_std_neg = E11_std_spot - E11_std_bar;
E11_std_negmidpos = transpose([E11_std_neg, E11_std_spot, E11_std_pos]);
E11_std_pos_neg = E11_std_pos - E11_std_neg;

E22_sem = std(E22, 0, 2) ./ sqrt(n);

S22_std_spot = S22_avg(4:2:end);
S22_std_spot_th = transpose([S22_std_spot S22_std_spot S22_std_spot]);
E22_std_spot = E22_avg(4:2:end);
E22_std_bar = E22_sem(4:2:end);

E22_std_pos = E22_std_spot + E22_std_bar;
E22_std_neg = E22_std_spot - E22_std_bar;
E22_std_negmidpos = transpose([E22_std_neg, E22_std_spot, E22_std_pos]);
E22_std_pos_neg = E22_std_pos - E22_std_neg;

% Figure with error bars
figure (1)
sz = 15;
plot(E11_avg, S11_avg, 'k', E22_avg, S22_avg, 'r', 'LineWidth', 2); hold on
scatter(E11_std_spot, S11_std_spot, sz, 'k'); hold on
plot(E11_std_negmidpos, S11_std_spot_th, 'k'); hold on
scatter(E22_std_spot, S22_std_spot, sz, 'r'); hold on
plot(E22_std_negmidpos, S22_std_spot_th, 'r');

% legend('Circumferential', 'Radial')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')

Savg_max = max(max(max(S11_avg)), (max(max(S22_avg))));  % max S for axis limits (y limit)
Estd_max = max(max(max(E11_std_negmidpos)), (max(max(E22_std_negmidpos))));
axis([0 Estd_max + 0.05 0 Savg_max + 10]);  % 1 for max S

set(gca, 'Ytick', 0:20:Savg_max + 10);
set(gca, 'Xtick', 0:0.1:Estd_max + 0.05);
set(findobj(gcf, 'type', 'axes'), 'FontName', 'Arial', 'FontSize', 18, 'FontWeight', 'Bold', 'LineWidth', 2);
box on;

x0 = 10;
y0 = 10;
width = 350;
height = 250;
set(gcf, 'units', 'points', 'position', [x0, y0, width, height])

% Figure without error bars
figure (2)
sz = 15;
scatter(E11_avg, S11_avg, sz, 'k'); hold on;
scatter(E22_avg, S22_avg, sz, 'r'); hold on;
Emax=max(max(max(E11_avg)),(max(max(E22_avg))));  % max E for axis limits (x limit)
Smax=max(max(max(S11_avg)),(max(max(S22_avg))));  % max S for axis limits (y limit)
axis([0 Emax+0.05 0 Smax+10]);  % add 0.1 for max E and 10 for max S

set(gca,'Ytick', 0:20:Smax+10);
set(gca,'Xtick', 0:0.1:Emax+0.05);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight',
'Bold', 'LineWidth', 2);
box on;

x0=10;
y0=10;
width=350;
height=250;
set(gcf,'units','points','position',[x0,y0,width,height])
%Plotting 2nd P-K stress vs Green strain

clc
clear all
close all

%Written by Jessica Perez
%Version April 2019

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%PI: Dr. Kartik Balachandran

%This code graphs all tension ratios in both circumferential and radial
direction with S vs E in one graph, E vs S and AS vs S in one direction,
and S11 vs S22

%Input:
% File containing ratio data

%Output:
% Graphs that need to be saved as jpeg or other filetype:
% (1) One graph with all ratios in both the circumferential and radial
direction for Green Strain (E) vs 2nd P-K stress (S)
% (2) One figure with 2 subplots. E vs 2nd P-K stress. Left graph is
% the circumferential direction and the right one is the radial
direction
% (3) One figure with 2 subplots. Areal strain (AS) vs 2nd P-K stress.
% Left graph is the circumferential direction and the right one is
% the radial direction
% (4) One graph with S11 vs S22 for all 5 ratios

%Note:
% All legends and axis can be uncommented to see appropriate symbols for
% each item graphed.

%To extract the files the data should be as follows on the file with
% all the ratio data:
% 1:1: Column A=E11; Column B=E22; Column C=S11; Column D=S22
% 1:0.75: Column I=E11; Column J=E22; Column K=S11; Column L=S22
% 0.75:1: Column Q=E11; Column R=E22; Column S=S11; Column T=S22
% 1:0.5: Column Y=E11; Column Z=E22; Column AA=S11; Column AB=S22
% 0.5:1: Column AG=E11; Column AH=E22; Column AI=S11; Column AJ=S22

%Extracting all ratio data from excel file
[filename,pathname]=uigetfile('*.*', 'Select file');
data=xlsread(filename);
index=size(data,1);

%Eseperating data by ratios 1:1, 1:0.75, 0.75:1, 1:0.5, 0.5:1
E11_1_1=data([1:index],1);
E22_1_1=data([1:index],2);
S11_1_1=data([1:index],3);
S22_1_1=data([1:index],4);
AS_1_1=data([1:index],7);
E11_1_75=data([1:index],9);
E22_1_75=data([1:index],10);
S11_1_75=data([1:index],11);
S22_1_75=data([1:index],12);
AS_1_75=data([1:index],15);
E11_75_1=data([1:index],17);
E22_75_1=data([1:index],18);
S11_75_1=data([1:index],19);
S22_75_1=data([1:index],20);
AS_75_1=data([1:index],23);
E11_1_50=data([1:index],25);
E22_1_50=data([1:index],26);
S11_1_50=data([1:index],27);
S22_1_50=data([1:index],28);
AS_1_50=data([1:index],31);
E11_50_1=data([1:index],33);
E22_50_1=data([1:index],34);
S11_50_1=data([1:index],35);
S22_50_1=data([1:index],36);
AS_50_1=data([1:index],39);
E11=[E11_1_1, E11_1_75, E11_75_1, E11_1_50, E11_50_1];
E22=[E22_1_1, E22_1_75, E22_75_1, E22_1_50, E22_50_1];
S11=[S11_1_1, S11_1_75, S11_75_1, S11_1_50, S11_50_1];
S22=[S22_1_1, S22_1_75, S22_75_1, S22_1_50, S22_50_1];
AS=[AS_1_1, AS_1_75, AS_75_1, AS_1_50, AS_50_1];

figure
figure(1)
sz=15;
scatter(E11_1_1,S11_1_1,sz,'k'); hold on;
scatter(E11_1_75,S11_1_75,sz,'r'); hold on;
scatter(E11_75_1,S11_75_1,sz,'g','v','filled','MarkerEdgeColor',[0 0 0],LineWidth',0.6); hold on;
scatter(E11_1_50,S11_1_50,sz,'y','^','filled','MarkerEdgeColor',[0 0 0],LineWidth',0.6); hold on;
scatter(E11_50_1,S11_50_1,sz,'b','s','filled','MarkerEdgeColor',[0 0 0],LineWidth',0.6); hold on;

sz=20;
scatter(E22_1_1,S22_1_1,sz,'k','x'); hold on;
scatter(E22_1_75,S22_1_75,sz,'r','x'); hold on;

sz=15;
scatter(E22_75_1,S22_75_1,sz,'g','d','filled','MarkerEdgeColor',[0 0 0],LineWidth',0.6); hold on;
scatter(E22_1_50,S22_1_50,sz,'y','d','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;
scatter(E22_50_1,S22_50_1,sz,'b','h','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;

title('All Ratios')
% legend('Circumferential 1:1','Circumferential 1:0.75','Circumferential 0.75:1','Circumferential 1:0.5','Radial 0.5:1','Radial 1:1','Radial 1:0.75','Radial 0.75:1','Radial 1:0.5')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')

Emax=max(max(max(E11)), (max(max(E22)))); % max E for axis limits (x limit)
Smax=max(max(max(S11)), (max(max(S22)))); % max S for axis limits (y limit)
axis([0 Emax+0.05 0 Smax+10]) % add 0.1 for max E and 10 for max S

set(gca, 'Ytick', 0:20:Smax+10);
set(gca, 'Xtick', 0:0.1:Emax+0.05);

box on;
x0=10;
y0=10;
width=350;
height=250;
set(gcf, 'units', 'points', 'position', [x0, y0, width, height])

figure (2)
subplot(1,2,1)
sz=15;
scatter(E11_1_1,S11_1_1,sz,'k'); hold on;
scatter(E11_1_75,S11_1_75,sz,'r'); hold on;
scatter(E11_75_1,S11_75_1,sz,'g','v','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;
scatter(E11_1_50,S11_1_50,sz,'y','^','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;
scatter(E11_50_1,S11_50_1,sz,'b','s','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;

Emax=max(max(E11)); % max E for axis limits (x limit)
Smax=max(max(S11)); % max S for axis limits (y limit)
axis([0 Emax+0.05 0 Smax+10]) % add 0.1 for max E and 10 for max S
set(gca, 'Ytick', 0:20:Smax+10);
set(gca, 'Xtick', 0:0.1:Emax+0.05);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold', 'LineWidth', 2);
box on;

figure(2)
subplot (1,2,2);
sz=20;
scatter(E22_1_1,S22_1_1,sz,'k','x'); hold on;
scatter(E22_1_75,S22_1_75,sz,'r','x'); hold on;

sz=15;
scatter(E22_75_1,S22_75_1,sz,'g','d','filled','MarkerEdgeColor',[0 0 0],'LineWidth',0.6); hold on;
scatter(E22_1_50,S22_1_50,sz,'y','d','filled','MarkerEdgeColor',[0 0 0],'LineWidth',0.6); hold on;
scatter(E22_50_1,S22_50_1,sz,'b','h','filled','MarkerEdgeColor',[0 0 0],'LineWidth',0.6); hold on;
title ('2nd P-K stress'); %title only so easier to save/distinguish
% legend('Radial 1:1','Radial 1:0.75','Radial 0.75:1','Radial 1:0.5')
% title('Radial')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')

Emax=max(max(E22)); %max E for axis limits (x limit)
Smax=max(max(S22)); %max S for axis limits (y limit)
axis([0 Emax+0.05 0 Smax+10]); % add 0.1 for max E and 10 for max S
set(gca,'Ytick', 0:20:Smax+10);
set(gca,'Xtick', 0:0.1:Emax+0.05);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold', 'LineWidth', 2);
box on;

x0=10;
y0=10;
width=800;
height=250;
set(gcf,'units','points','position',[x0,y0,width,height])

figure (3)
subplot(1,2,1)
sz=15;
scatter(AS_1_1,S11_1_1,sz,'k'); hold on;
scatter(AS_1_75,S11_1_75,sz,'r'); hold on;
scatter(AS_75_1,S11_75_1,sz,'g','v','filled','MarkerEdgeColor',[0 0 0],'LineWidth',0.6); hold on;
scatter(AS_1_50,S11_1_50,sz,'y','^','filled','MarkerEdgeColor',[0 0 0],'LineWidth',0.6); hold on;
scatter(AS_50_1,S11_50_1,sz,'b','s','filled','MarkerEdgeColor',[0 0 0],'LineWidth',0.6); hold on;
title ('Areal Strain vs.'); %title only so easier to save/distinguish
ASmax=max(max(AS)); % max E for axis limits (x limit)
Smax=max(max(S11)); % max S for axis limits (y limit)
axis([0 ASmax+0.02 0 Smax+10]); % add 0.1 for max E and 10 for max S
set(gca,'Ytick', 0:20:Smax+10);
set(gca,'Xtick', 0:0.05:ASmax+0.02);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,' FontWeight', 'Bold', 'LineWidth', 2);
box on;

figure(3)
subplot (1,2,2);
sz=20;
scatter(AS_1_1,S22_1_1,sz,'k','x'); hold on;
scatter(AS_1_75,S22_1_75,sz,'r','x'); hold on;

sz=15;
scatter(AS_75_1,S22_75_1,sz,'g','d','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;
scatter(AS_1_50,S22_1_50,sz,'y','d','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;
scatter(AS_50_1,S22_50_1,sz,'b','h','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;
title ('2nd P-K stress'); % title only so easier to save/distinguish

ASmax=max(max(AS)); % max E for axis limits (x limit)
Smax=max(max(S22)); % max S for axis limits (y limit)
axis([0 ASmax+0.02 0 Smax+10]); % add 0.1 for max E and 10 for max S
set(gca,'Ytick', 0:20:Smax+10);
set(gca,'Xtick', 0:0.05:ASmax+0.02);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,' FontWeight', 'Bold', 'LineWidth', 2);
box on;
x0=10;
y0=10;
width=800;
height=250;
set(gcf,'units','points','position',[x0,y0,width,height])

figure(4)
scatter(S11_1_1,S22_1_1,sz,'k'); hold on;
scatter(S11_1_75,S22_1_75,sz,'r'); hold on;
scatter(S11_75_1,S22_75_1,sz,'g','v','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;
scatter(S11_1_50,S22_1_50,sz,'y','^','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;
scatter(S11_50_1,S22_50_1,sz,'b','s','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;

title ('2nd P-K stress circ vs rad'); %title only so easier to save/distinguish
% legend('1:1','1:0.75','0.75:1','1:0.5')
% title('Radial')
% xlabel('S22 (kPa)')
% ylabel('S22 (kPa)')

S11max=max(max(S11)); %max S11 for axis limits (x limit)
S22max=max(max(S22)); %max S22 for axis limits (y limit)
axis([0 S11max+10 0 S22max+10]); % add 0.1 for max E and 10 for max S
set(gca,'Ytick', 0:20:S22max+10);
set(gca,'Xtick', 0:20:S11max+10);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold', 'LineWidth', 2);
box on;

x0=10;
y0=10;
width=350;
height=250;
set(gcf,'units','points','position',[x0,y0,width,height])
Constitutive Models MATLAB Instructions and Codes

For the constitutive models, excel sheets containing the Green strain (E), 1st P-K (P) stress, and 2nd P-K (S) stress in the circumferential and radial direction of all tension loading ratios must be made. MATLAB code Biaxial_4.m exports an excel sheet (plotdata.xlsx) containing E, S, and P in both the circumferential and radial direction and the areal strain for each tension loading ratios. One excel sheet needs to be made containing all 5 ratios (1:1, 1:0.75, 0.75:1, 1:0.50, and 0.50:1).

The columns would be as followed. For the first tension ratio of 1:1: column A: E11; column B: E22; column C: S11; column D: S22; column E: P11; column F: P22, column G: AS, and column H: empty. Repeat in the preceding columns for the remaining ratios in the order of 1:1, 1:0.75, 0.75:1, 1:0.50, and 0.50:1 i.e. Tension ratio 1:0.75: column I: E11; column J: E22, etc. Tension ratio 0.75:1: column Q: E11; column R: E22, etc. Tension ratio 1:0.50: column Y: E11; column Z: E22, etc. Tension ratio 0.50:1: column AG: E11; column AH: E22, etc. It is important to create the excel file in this order as MATLAB codes load the data accordingly.
%Isotropic Modified Mooney-Rivlin
clc
clear all
close all
warning off;

%Written by Jessica Perez
%Version January 2019

%University of Arkansas
%Department of Biomedical Engineering
%Mechanobiology and Soft Materials Laboratory (MSML)
%PI: Dr. Kartik Balachandran

%This code calculates the constants for the Mooney Rivlin computational
%model and graphs the experimental data and model data.

%%Yang Modified Mooney-Rivlin-Isotropic Equation

%Strain Energy (W)
&W=c1*(I1-3)+c2*(I2-3)+D1*[exp(D2*(I1-3))-1]

%Cauchy Stress (sigma)
%sigma=-p*I+2.*dW/dI1.*B-2.*dW/dI2.*B.^-1
%p=hydrostatic pressure term associated with the incompressibility
%constraint

%I1 and I2 from Kanner to use with Yang
%I1=trB=lam1^2+lam2^2+lam3^2
%I2=1/2*[(trB)^2)-(tr(B^2))]=lam1^2*lam2^2+lam2^2*lam3^2+lam3^2*lam1^2

%E vs S
%S11=(2E11+1)^(-1)*[2c2-2(c1+D1[D2exp(D2(2E11+2E22+3)-3D2)))+2(2E11+1)
%+(c1+D1[D2exp(D2(2E11+2E22+3)-3D2))]-2c2(2E11+1)^(-1)]
%S22=(2E22+1)^(-1)*[2c2-2(c1+D1[D2exp(D2(2E11+2E22+3)-3D2)))+2(2E22+1)
%+(c1+D1[D2exp(D2(2E11+2E22+3)-3D2))]-2c2(2E22+1)^(-1)]

%Note:
%To extract the files the data should be as follows on the file with
%all the ratio data.
%1:1: Column A=E11; Column B=E22; Column C=S11; Column D=S22
%1:0.75: Column I=E11; Column J=E22; Column K=S11; Column L=S22
%0.75:1: Column Q=E11; Column R=E22; Column S=S11; Column T=S22
%1:0.5: Column Y=E11; Column Z=E22; Column AA=S11; Column AB=S22
%0.5:1: Column AG=E11; Column AH=E22; Column AI=S11; Column AJ=S22

%Input:
%File containing ratio data

%Output:
%Excel file:Mooney-Rivlin Constants.xlsx
% Contains: c1,c2,D1,D2,condition data, r square combined
% Graphs that need to be saved as jpeg or other filetype:
% (1) Individual ratios of the circumferential and radial together (5
% figures in subplot)
% Legend:
%   Experimental Circumferential=black circles
%   Experimental Radial=red circles
%   Model Fit=Red Line
% Order:
%     left to right= 1:1, 1:0.75, 0.75:1, 1:0.5, 0.5:1
% (2) All ratios with circumferential and radial separate (2 figures in
% subplot)
% Legend:
%   Experimental 1:1=black circles
%   Experimental 1:0.75=red circles
%   Experimental 0.75:1=upside down green triangle
%   Experimental 1:0.50=yellow triangle
%   Experimental 0.5:1=blue square
%   Model Fit=Red Line
% Order:
%     left to right= Circumferential, Radial

% Note:
% (1)Figures are the size that allows the subplots to be saved
% properly and not be stretched out.
% (2)Figures have no titles, x and y labels, or legends. I put those when
% I'm compiling figures in a powerpoint. However, the code to do so is
% commented so you can just uncomment them for whatever you want on
% the graph.

%Extracting all ratio data from excel file
[filename,pathname]=uigetfile('*.*', 'Select file');
data=xlsread(filename);
index=size(data,1);

%Seperating data by ratios 1:1, 1:0.75, 0.75:1, 1:0.5, 0.5:1
E11_1_1=data([1:index],1);
E22_1_1=data([1:index],2);
S11_1_1=data([1:index],3);
S22_1_1=data([1:index],4);
P11_1_1=data([1:index],5);
P22_1_1=data([1:index],6);
AS_1_1=data([1:index],7);

E11_1_75=data([1:index],9);
E22_1_75=data([1:index],10);
S11_1_75=data([1:index],11);
S22_1_75=data([1:index],12);
P11_1_75=data([1:index],13);
P22_1_75=data([1:index],14);
AS_1_75=data([1:index],15);

E11_75_1=data([1:index],17);
E22_75_1=data([1:index],18);
S11_75_1=data([1:index],19);
S22_75_1=data([1:index],20);
P11_75_1=data([1:index],21);
P22_75_1=data([1:index],22);
AS_75_1=data([1:index],23);

E11_1_50=data([1:index],25);
E22_1_50=data([1:index],26);
S11_1_50=data([1:index],27);
S22_1_50=data([1:index],28);
P11_1_50=data([1:index],29);
P22_1_50=data([1:index],30);
AS_1_50=data([1:index],31);

E11_50_1=data([1:index],33);
E22_50_1=data([1:index],34);
S11_50_1=data([1:index],35);
S22_50_1=data([1:index],36);
P11_50_1=data([1:index],37);
P22_50_1=data([1:index],38);
AS_50_1=data([1:index],39);
E11_1=data([1:index],1);
E22_1=data([1:index],2);
S11_1=data([1:index],3);
S22_1=data([1:index],4);

E11_1_75=data([1:index],9);
E22_1_75=data([1:index],10);
S11_1_75=data([1:index],11);
S22_1_75=data([1:index],12);

E11_75_1=data([1:index],17);
E22_75_1=data([1:index],18);
S11_75_1=data([1:index],19);
S22_75_1=data([1:index],20);

E11_1_50=data([1:index],25);
E22_1_50=data([1:index],26);
S11_1_50=data([1:index],27);
S22_1_50=data([1:index],28);

E11_50_1=data([1:index],33);
E22_50_1=data([1:index],34);
S11_50_1=data([1:index],35);
S22_50_1=data([1:index],36);

% Model formulation

syms c1 c2 D1 D2 E11 E22 real
W=2.*c1.*(E11+E22)+4.*(E11.*E22+E11+E22)+D1.*(exp(2.*D2.*(E11+E22))-1);
fun_11=matlabFunction(diff(W,E11)); %symbolic
fun_22=matlabFunction(diff(W,E22)); %symbolic
clear E11 E22

% Objective function. This is the sum of squares of the residuals. I had to
% write out the complete equations because I'm not sure yet how to
% incorporate function handles fun_11 and fun_22 into this.
obj_fun = @(x) ... + sum(((2.*E11_1_1+1).^(-1).*(2.*x(2)-2.*(x(1)+x(3).*(x(4).*exp(x(4))).*(2.*E11_1_1+2.*E22_1_1+3-3.*x(4))))+2.*(2.*E11_1_1+1.)*(x(1)+x(3).*(x(4).*exp(x(4))).*(2.*E11_1_1+2.*E22_1_1+3-3.*x(4))))-2.*x(2).*(2.*E11_1_1+1.).^(-1)))-S11_1_1.^(2)+((2.*E22_1_1+1.).^(-1).*(2.*x(2)-2.*(x(1)+x(3).*(x(4).*exp(x(4))).*(2.*E11_1_1+2.*E22_1_1+3-3.*x(4))))+2.*(2.*E22_1_1+1.)*(x(1)+x(3).*(x(4).*exp(x(4))).*(2.*E11_1_1+2.*E22_1_1+3-3.*x(4))))-2.*x(2).*(2.*E22_1_1+1.).^(-1)))-S22_1_1.^(2));

% obj_fun=matlabFunction(sum((sym(fun_11)-S11).^2+(sym(fun_22)-S22).^2));

% Minimization of objective function using fmincon
x0=[1,2,3,4]; % initial guesses
lb=[-inf,-inf,-inf,-inf]; % lower bound for parameters
ub=[inf,inf,inf,inf]; % upper bound for parameters
A=[0,0,0,0;0,0,0,0;0,0,0,0;0,0,0,0]; % Variables to enforce inequality constraint
b=[0,0,0,0]; % Variables to enforce inequality constraint
options = optimoptions('fmincon','Display','iter','Algorithm','sqp'); %Options to control execution of fmincon
options = optimoptions('fmincon','Display','iter','Algorithm','sqp','MaxIterations',10000,'MaxFunEval',100000,'StepTolerance',1e-20);
x = fmincon(obj_fun,x0,A,b,[],[],lb,ub,[],options);

% Backed out coefficients
c1=x(1)
c2=x(2)
D1=x(3)
D2=x(4)

% Matrix form of coefficients
D=[c1,c2,D1,D2];

% Total R_square
SSres=...
   + sum(((2.*E11_1_1+1).^(-1).*(2.*x(2)-
2.*(x(1)+x(3)).*(x(4)).*exp(x(4)).*(2.*E11_1_1+2.*E22_1_1+3.*x(4)))))+2.*(2.*E11_1_1+1).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_1+2.*E22_1_1+3.*x(4)))-2.*x(2).*E11_1_1.^(-1))-
S11_1_1.^2+(2.*E22_1_1+1).^(-1).*2.*x(2)-
2.*(x(1)+x(3)).*(x(4)).*exp(x(4)).*(2.*E11_1_1+2.*E22_1_1+3.*x(4)))+2.*(2.*E11_1_1+1).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_1+2.*E22_1_1+3.*x(4)))-2.*x(2).*E11_1_1.^(-1)))-S22_1_1.^2; (2)... + sum(((2.*E11_1_75+1).^(-1).*2.*x(2))-2.*(x(1)+x(3)).*(x(4)).*exp(x(4)).*(2.*E11_1_75+2.*E22_1_75+3.*x(4)))+2.*(2.*E22_1_75+1).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_75+2.*E22_1_75+3.*x(4)))-2.*x(2).*E11_1_75+1.^(-1)))-S11_1_75.^2+(2.*E22_1_75+1).^(-1).*2.*x(2)-2.*(x(1)+x(3)).*(x(4)).*exp(x(4)).*(2.*E11_1_75+2.*E22_1_75+3.*x(4)))+2.*(2.*E22_1_75+1).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_75+2.*E22_1_75+3.*x(4)))-2.*x(2).*E11_1_75+1.^(-1)))-S22_1_75.^2; (2)... + sum(((2.*E11_1_50+1).^(-1).*2.*x(2))-2.*(x(1)+x(3)).*(x(4)).*exp(x(4)).*(2.*E11_1_50+2.*E22_1_50+3.*x(4)))+2.*(2.*E22_1_50+1).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_50+2.*E22_1_50+3.*x(4)))-2.*x(2).*E11_1_50+1.^(-1)))-S11_1_50.^2+(2.*E22_1_50+1).^(-1).*2.*x(2)-2.*(x(1)+x(3)).*(x(4)).*exp(x(4)).*(2.*E11_1_50+2.*E22_1_50+3.*x(4)))+2.*(2.*E22_1_50+1).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_50+2.*E22_1_50+3.*x(4)))-2.*x(2).*E11_1_50+1.^(-1)))-S22_1_50.^2; (2)... + sum(((2.*E11_1_50+1).^(-1).*2.*x(2))-2.*(x(1)+x(3)).*(x(4)).*exp(x(4)).*(2.*E11_1_50+2.*E22_1_50+3.*x(4)))+2.*(2.*E22_1_50+1).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_50+2.*E22_1_50+3.*x(4)))-2.*x(2).*E11_1_50+1.^(-1)))-S11_1_50.^2+(2.*E22_1_50+1).^(-1).*2.*x(2)-2.*(x(1)+x(3)).*(x(4)).*exp(x(4)).*(2.*E11_1_50+2.*E22_1_50+3.*x(4)))+2.*(2.*E22_1_50+1).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_50+2.*E22_1_50+3.*x(4)))-2.*x(2).*E11_1_50+1.^(-1)))-S22_1_50.^2; (2));

c_bar1=mean2(S11_1_1);
r_bar1=mean2(S22_1_1);
c_bar2=mean2(S11_1_75);
r_bar2=mean2(S22_1_75);
c_bar3=mean2(S11_75_1);
r_bar3=mean2(S22_75_1);
c_bar4=mean2(S11_1_50);
r_bar4=mean2(S22_1_50);
c_bar5=mean2(S11_50_1);
r_bar5=mean2(S22_50_1);

$S_{Stot} = \text{...}$
$+ \text{sum(((S11_1_1-c_bar1).^2)+((S22_1_1-r_bar1).^2))...}$
$+ \text{sum(((S11_1_75-c_bar2).^2)+((S22_1_75-r_bar2).^2))...}$
$+ \text{sum(((S11_75_1-c_bar3).^2)+((S22_75_1-r_bar3).^2))...}$
$+ \text{sum(((S11_1_50-c_bar4).^2)+((S22_1_50-r_bar4).^2))...}$
$+ \text{sum(((S11_50_1-c_bar5).^2)+((S22_50_1-r_bar5).^2));}$

$R_{square\_combine}=1-(SS_{res}/SS_{tot})$

%Export constants into excel sheet
output=[c1,c2,D1,D2, R_square_combine];
h={'c1', 'c2', 'D1', 'D2', 'R_square_combine'};
m=[h;num2cell(output)];
filename='Modified M-R (Isotropic) Constants.xlsx';
xlsxwrite(filename,m);

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Generated values for plotting model fit
E11_1_1_m=linspace(0,max(E11_1_1),1000);
E22_1_1_m=linspace(0,max(E22_1_1),1000);
model11_1_1_m=(2.*E11_1_1_m+1).^(-1).*(2.*x(2)-2.*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_1_m+2.*E22_1_1_m+3)-3.*x(4)))+2.*x(2).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_1_m+2.*E22_1_1_m+3)-3.*x(4));
model22_1_1_m=(2.*E22_1_1_m+1).^(-1).*(2.*x(2)-2.*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_1_m+2.*E22_1_1_m+3)-3.*x(4)))+2.*x(2).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_1_m+2.*E22_1_1_m+3)-3.*x(4));

E11_1_75_m=linspace(0,max(E11_1_75),1000);
E22_1_75_m=linspace(0,max(E22_1_75),1000);
model11_1_75_m=(2.*E11_1_75_m+1).^(-1).*(2.*x(2)-2.*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_75_m+2.*E22_1_75_m+3)-3.*x(4)))+2.*x(2).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_75_m+2.*E22_1_75_m+3)-3.*x(4));
model22_1_75_m=(2.*E22_1_75_m+1).^(-1).*(2.*x(2)-2.*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_75_m+2.*E22_1_75_m+3)-3.*x(4)))+2.*x(2).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_1_75_m+2.*E22_1_75_m+3)-3.*x(4));

E11_75_1_m=linspace(0,max(E11_75_1),1000);
E22_75_1_m=linspace(0,max(E22_75_1),1000);
model11_75_1_m=(2.*E11_75_1_m+1).^(-1).*(2.*x(2)-2.*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_75_1_m+2.*E22_75_1_m+3)-3.*x(4)))+2.*x(2).*x(1)+x(3).*x(4).*exp(x(4)).*(2.*E11_75_1_m+2.*E22_75_1_m+3)-3.*x(4));
\(3.\times(x(4))) + 2.\times(2.\times E_{11\_75\_1\_m} + 2.\times E_{22\_75\_1\_m} - 3.\times(x(4))) - 2.\times(2.\times E_{11\_75\_1\_m} + 2.\times E_{22\_75\_1\_m})\)^{-(1)}; 

\(\text{model}_{22\_75\_1\_m} = (2.\times E_{22\_75\_1\_m} + 1)^{-(1)} \times (x(1) + x(3) \times (x(4) \times \exp(x(4) \times (2.\times E_{11\_75\_1\_m} + 2.\times E_{22\_75\_1\_m} + 3) - 3.\times x(4)))) + 2.\times(2.\times E_{22\_75\_1\_m} + 1) \times (x(1) + x(3) \times (x(4) \times \exp(x(4) \times (2.\times E_{11\_75\_1\_m} + 2.\times E_{22\_75\_1\_m} + 3) - 3.\times x(4)))) - 2.\times(2.\times E_{22\_75\_1\_m} + 1)^{-(1)}; 

\(\text{model}_{11\_75\_1\_m} = \text{linspace}(0, \max(\text{model}_{11\_75\_1\_m}), 1000); \)

\(\text{model}_{22\_75\_1\_m} = \text{linspace}(0, \max(\text{model}_{22\_75\_1\_m}), 1000); \)

\(\text{model}_{11\_50\_1\_m} = \text{linspace}(0, \max(\text{model}_{11\_50\_1\_m}), 1000); \)

\(\text{model}_{22\_50\_1\_m} = \text{linspace}(0, \max(\text{model}_{22\_50\_1\_m}), 1000); \)

\(\text{E}_{11\_50\_1\_m} = \text{linspace}(0, \max(\text{E}_{11\_50\_1\_m}), 1000); \)

\(\text{E}_{22\_50\_1\_m} = \text{linspace}(0, \max(\text{E}_{22\_50\_1\_m}), 1000); \)

\(\text{S}_{11\_50\_1\_m} = \text{linspace}(0, \max(\text{S}_{11\_50\_1\_m}), 1000); \)

\(\text{S}_{22\_50\_1\_m} = \text{linspace}(0, \max(\text{S}_{22\_50\_1\_m}), 1000); \)

\(\text{model}_{11\_50\_1\_m} = \text{linspace}(0, \max(\text{model}_{11\_50\_1\_m}), 1000); \)

\(\text{model}_{22\_50\_1\_m} = \text{linspace}(0, \max(\text{model}_{22\_50\_1\_m}), 1000); \)

\%Plot:

\% Large matrix containg all values
\text{E}_{11\_1\_1\_m} = [\text{E}_{11\_1\_1\_m}, \text{E}_{11\_1\_75\_m}, \text{E}_{11\_75\_1\_m}, \text{E}_{11\_75\_1\_m}, \text{E}_{11\_50\_1\_m}, \text{E}_{11\_50\_1\_m}];
\text{E}_{22\_1\_1\_m} = [\text{E}_{22\_1\_1\_m}, \text{E}_{22\_1\_75\_m}, \text{E}_{22\_75\_1\_m}, \text{E}_{22\_75\_1\_m}, \text{E}_{22\_50\_1\_m}, \text{E}_{22\_50\_1\_m}];
\text{S}_{11\_1\_1\_m} = [\text{S}_{11\_1\_1\_m}, \text{S}_{11\_1\_75\_m}, \text{S}_{11\_75\_1\_m}, \text{S}_{11\_75\_1\_m}, \text{S}_{11\_50\_1\_m}, \text{S}_{11\_50\_1\_m}];
\text{S}_{22\_1\_1\_m} = [\text{S}_{22\_1\_1\_m}, \text{S}_{22\_1\_75\_m}, \text{S}_{22\_75\_1\_m}, \text{S}_{22\_75\_1\_m}, \text{S}_{22\_50\_1\_m}, \text{S}_{22\_50\_1\_m}];
\text{model}_{11\_1\_1\_m} = [\text{model}_{11\_1\_1\_m}, \text{model}_{11\_1\_75\_m}, \text{model}_{11\_75\_1\_m}, \text{model}_{11\_75\_1\_m}, \text{model}_{11\_50\_1\_m}, \text{model}_{11\_50\_1\_m}];
\text{model}_{22\_1\_1\_m} = [\text{model}_{22\_1\_1\_m}, \text{model}_{22\_1\_75\_m}, \text{model}_{22\_75\_1\_m}, \text{model}_{22\_75\_1\_m}, \text{model}_{22\_50\_1\_m}, \text{model}_{22\_50\_1\_m}];

\%Figure (1) plots Individual ratios of the circumferential and radial %together (5 figures in subplot) Figure is very wide to insure that plots %are displayed properly. Save figure as jpeg or desired file type.

\%Size of markers in scatterplot
\text{sz}=15;

\text{figure (1)
subplots (1,5,1)
scatter(E11_1_1,S11_1_1,sz,'k'); hold on;
scatter(E22_1_1,S22_1_1,sz,'r'); hold on;
plot(E11_1_1_m,model11_1_1_m,'r',E22_1_1_m,model22_1_1_m,'r'); hold off;
Emax_1_1=max(max(max(E11_1_1)),(max(max(E22_1_1)))); %max E for axis limits (x limit)
Smax_1_1=max(max(max(S11_1_1)),(max(max(S22_1_1)))); %max S for axis limits (y limit)
axis([0 Emax_1_1+0.05 0 Smax_1_1+10]) % add 0.1 for max E and 10 for max S
% legend('Experimental-Circumferential','S22-Experimental-Radial','Model Fit-Circumferential','Model Fit-Radial')
% title('1:1')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
set(gca,'Ytick', 0:20:Smax_1_1+10);
set(gca,'Xtick', 0:0.1:Emax_1_1+0.05);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold', 'LineWidth', 2);
box on;

figure (1)
subplots (1,5,2)
scatter(E11_1_75,S11_1_75,sz,'k'); hold on;
scatter(E22_1_75,S22_1_75,sz,'r'); hold on;
plot(E11_1_75_m,model11_1_75_m,'r',E22_1_75_m,model22_1_75_m,'r'); hold off;
Emax_1_75=max(max(max(E11_1_75)),(max(max(E22_1_75)))); %max E for axis limits (x limit)
Smax_1_75=max(max(max(S11_1_75)),(max(max(S22_1_75)))); %max S for axis limits (y limit)
axis([0 Emax_1_75+0.05 0 Smax_1_75+10]) % add 0.1 for max E and 10 for max S
% legend('Experimental-Circumferential','S22-Experimental-Radial','Model Fit-Circumferential','Model Fit-Radial')
% title('0.75:1')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
set(gca,'Ytick', 0:20:Smax_1_75+10);
set(gca,'Xtick', 0:0.1:Emax_1_75+0.05);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold', 'LineWidth', 2);
box on;

figure (1)
subplots (1,5,3)
sz=15;
scatter(E11_75_1,S11_75_1,sz,'k'); hold on;
scatter(E22_75_1,S22_75_1,sz,'r'); hold on;
plot(E11_75_1_m,model11_75_1_m,'r',E22_75_1_m,model22_75_1_m,'r'); hold off;
Emax_75_1=max(max(max(E11_75_1)),(max(max(E22_75_1)))); %max E for axis limits (x limit)
Smax_75_1=max(max(max(S11_75_1)),(max(max(S22_75_1)))); %max S for axis limits (y limit)
axis([0 Emax_75_1+0.05 0 Smax_75_1+10]) % add 0.1 for max E and 10 for max S
% legend('Experimental-Circumferential','S22-Experimental-Radial','Model Fit-Circumferential','Model Fit-Radial')
figure (1)
subplot (1,5,1)
saveas(gcf,'./pic1_50.jpg');

title('0.5:1')
xlabel('Green Strain')
ylabel('2nd P-K Stress (kPa)')
set(gca,'Ytick',0:20:Smax_50+10);
set(gca,'Xtick',0:0.1:Emax_50+0.05);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold','LineWidth',2);
box on;

Emax_50=max(max(max(E11_50)),(max(max(E22_50)))); %max E for axis limits (x limit)
Smax_50=max(max(max(S11_50)),(max(max(S22_50)))); %max S for axis limits (y limit)
axis([0 Emax_50+0.05 0 Smax_50+10]) % add 0.1 for max E and 10 for max S
% legend('Experimental-Circumferential','S22-Experimental-Radial','Model Fit-Circumferential','Model Fit-Radial')
% title('0.5:1')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
set(gca,'Ytick',0:20:Smax_50+10);
set(gca,'Xtick',0:0.1:Emax_50+0.05);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold','LineWidth',2);
box on;

x0=10;
y0=10;
width=1600;


height=200;
set(gcf,'units','points','position',[x0,y0,width,height])

%Figure (2) plots all ratios with circumferential and radial separate
%(2 figures in subplot). Save figure as jpeg or desired file type.

%Note: label of each ratio-1:1=circle; 1:0.75=astrix
figure (2)
subplot (1,2,1)
scatter(E11_1_1,S11_1_1,sz,'k'); hold on;
scatter(E11_1_75,S11_1_75,sz,'r'); hold on;
scatter(E11_75_1,S11_75_1,sz,'g','v','filled','MarkerEdgeColor',[0 0 0],'
LineWidth',0.6); hold on;
scatter(E11_1_50,S11_1_50,sz,'y','^','filled','MarkerEdgeColor',[0 0 0],'
LineWidth',0.6); hold on;
scatter(E11_50_1,S11_50_1,sz,'b','s','filled','MarkerEdgeColor',[0 0 0],'
LineWidth',0.6); hold on;
plot(E11_1_1_m,model11_1_1_m,'r',E11_1_75_m,model11_1_75_m,'r',E11_75_1_m,
model11_75_1_m,'r',E11_1_50_m,model11_1_50_m,'r',E11_50_1_m,model11_50_1_m,'r');
hold off;

Emax=max(max(E11)); %max E for axis limits (x limit)
Smax=(max(max(max(S11)),(max(model11)))); %max S for axis limits (x limit)
axis([0 Emax+0.05 0 Smax+10]); % add 0.1 for max E and 10 for max S
legend('Experimental 1:1','Experimental 1:0.75','Experimental
0.75:1','Experimental 1:0.5','Experimental 0.5:1','Model Fit 1:1','Model Fit
1:0.75','Model Fit 0.75:1','Model Fit 1:0.5','Model Fit 0.5:1')
% title('Circumferential')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
set(gca,'Ytick', 0:20:Smax+10);
set(gca,'Xtick', 0:0.1:Emax+0.05);

figure (2)
subplot (1,2,2)
scatter(E22_1_1,S22_1_1,sz,'k'); hold on;
scatter(E22_1_75,S22_1_75,sz,'r'); hold on;
scatter(E22_75_1,S22_75_1,sz,'g','v','filled','MarkerEdgeColor',[0 0 0],'
LineWidth',0.6); hold on;
scatter(E22_1_50,S22_1_50,sz,'y','^','filled','MarkerEdgeColor',[0 0 0],'
LineWidth',0.6); hold on;
scatter(E22_50_1,S22_50_1,sz,'b','s','filled','MarkerEdgeColor',[0 0 0],'
LineWidth',0.6); hold on;
plot(E22_1_1_m,model22_1_1_m,'r',E22_1_75_m,model22_1_75_m,'r',E22_75_1_m,
model22_75_1_m,'r',E22_1_50_m,model22_1_50_m,'r',E22_50_1_m,model22_50_1_m,'r');
hold off;

Emax=max(max(E22)); %max E for axis limits (x limit)
Smax=(max(max(max(S22)),(max(model22)))); %max S for axis limits (y limit)
axis([0 Emax+0.05 0 Smax+10]); % add 0.1 for max E and 10 for max S
set(gca,'Ytick', 0:20:Smax+10);
set(gca,'Xtick', 0:0.1:Emax+0.05);
%legend('Experimental 1:1','Experimental 1:0.75','Experimental 0.75:1','Experimental 1:0.5','Experimental 0.5:1','Model Fit 1:1','Model Fit 1:0.75','Model Fit 0.75:1','Model Fit 1:0.5','Model Fit 0.5:1')
% title('Radial')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',12,'FontWeight','Bold','LineWidth',2);
box on;

x0=10;
y0=10;
width=800;
height=250;
set(gcf,'units','points','position',[x0,y0,width,height])
% Anisotropic Modified Mooney-Rivlin

clc
clear all
close all
warning off;

% Written by Jessica Perez
% Version January 2019

% University of Arkansas
% Department of Biomedical Engineering
% Mechanobiology and Soft Materials Laboratory (MSML)
% PI: Dr. Kartik Balachandran

% This code calculates the constants for the Mooney Rivlin computational
% model and graphs the experimental data and model data.

% Rassouli Mooney Rivlin Modified Anisotropic Equation

% Strain Energy (W)
W = c1(I1 - 3) + D1[exp(D2(I1 - 3)) - 1] + (k1/2k2)[exp[k2(I4 - 1)^2] - 1]

% Cauchy Stress (sigma)
sigma = -pI + 2.*dW/dI1.*B - 2.*dW/dI2.*B.^-1
p = hydrostatic pressure term associated with the incompressibility constraint

% I1 and I2 from Kanner
I1 = trB = lam1^2 + lam2^2 + lam3^2
I2 = 1/2*[(trB)^2 - tr(B^2)] = lam1^2*lam2^2 + lam2^2*lam3^2 + lam3^2*lam1^2
I4 = Cij(nc)i(nc)j

% E vs S
S11 = [4E11(c1 + D1D2exp(2E11 + 2E22)) + 4k1E11(2E11 + 1)exp(k2(2E11)^2)]/(2E11 + 1)
S22 = [4E22(c1 + D1D2exp(2E11 + 2E22))]/(2E22 + 1)

% Note:
% To extract the files the data should be as follows on the file with
% all the ratio data.
% 1:1: Column A=E11; Column B=E22; Column C=S11; Column D=S22
% 1:0.75: Column I=E11; Column J=E22; Column K=S11; Column L=S22
% 0.75:1: Column Q=E11; Column R=E22; Column S=S11; Column T=S22
% 1:0.5: Column Y=E11; Column Z=E22; Column AA=S11; Column AB=S22
% 0.5:1: Column AG=E11; Column AH=E22; Column AI=S11; Column AJ=S22

% Input:
% File containing ratio data

% Output:
% Excel file: Mooney-Rivlin Constants.xlsx
% Contains: c1,c2,D1,D2, condition data, r square combined
% Graphs that need to be saved as jpeg or other filetype:
%   (1) Individual ratios of the circumferential and radial together (5
%       figures in subplot)
%     Legend:
%       Experimental Circumferential=black circles
%       Experimental Radial=red circles
%       Model Fit=Red Line
%     Order:
%       left to right= 1:1, 1:0.75, 0.75:1, 1:0.5, 0.5:1
%   (2) All ratios with circumferential and radial seperate (2 figures in
%       subplot)
%     Legend:
%       Experimental 1:1=black circles
%       Experimental 1:0.75=red circles
%       Experimental 0.75:1=upside down green triangle
%       Experimental 1:0.50=yellow triangle
%       Experimental 0.5:1=blue square
%       Model Fit=Red Line
%     Order:
%       left to right= Circumferential, Radial

% Note:
%   (1) Figures are the size that allows the subplots to be saved
%       properly and not be stretched out.
%   (2) Figures have no titles, x and y labels, or legends. I put those when
%       I'm compiling figures in a powerpoint. However, the code to do so is
%       commented so you can just uncomment them for whatever you want on
%       the graph.

% Extracting all ratio data from excel file
[filename,pathname]=uigetfile('*.xlsx', 'Select file');
data=xlsread(filename);
index=size(data,1);

% Separating data by ratios 1:1, 1:0.75, 0.75:1, 1:0.5, 0.5:1
E11_1_1=data([1:index],1);
E22_1_1=data([1:index],2);
S11_1_1=data([1:index],3);
S22_1_1=data([1:index],4);

E11_1_75=data([1:index],9);
E22_1_75=data([1:index],10);
S11_1_75=data([1:index],11);
S22_1_75=data([1:index],12);

E11_75_1=data([1:index],17);
E22_75_1=data([1:index],18);
S11_75_1=data([1:index],19);
S22_75_1=data([1:index],20);

E11_1_50=data([1:index],25);
E22_1_50=data([1:index],26);
S11_1_50=data([1:index],27);
S22_1_50=data([1:index],28);
% Objective function. This is the sum of squares of the residuals.
obj_fun = @(x) ... 
+ sum(((4.*E11_1_1.*(x(1)+x(2).*x(3).*exp(2.*E11_1_1+2.*E22_1_1))+4.*x(4).*E11_1_1.*(2.*E11_1_1+1).*exp(x(5).*((2.*E11_1_1).^2))./(2.*E11_1_1+1)) - S11_1_1).^2 + (((4.*E22_1_1.*(x(1)+x(2).*x(3).*exp(2.*E11_1_1+2.*E22_1_1)))./(2.*E22_1_1+1)) - S22_1_1).^2) ... 
+ sum(((4.*E11_1_75.*(x(1)+x(2).*x(3).*exp(2.*E11_1_75+2.*E22_1_75))+4.*x(4).*E11_1_75.*(2.*E11_1_75+1).*exp(x(5).*((2.*E11_1_75).^2))./(2.*E11_1_75+1)) - S11_1_75).^2 + (((4.*E22_1_75.*(x(1)+x(2).*x(3).*exp(2.*E11_1_75+2.*E22_1_75)))./(2.*E22_1_75+1)) - S22_1_75).^2) ... 
+ sum(((4.*E11_1_50.*(x(1)+x(2).*x(3).*exp(2.*E11_1_50+2.*E22_1_50))+4.*x(4).*E11_1_50.*(2.*E11_1_50+1).*exp(x(5).*((2.*E11_1_50).^2))./(2.*E11_1_50+1)) - S11_1_50).^2 + (((4.*E22_1_50.*(x(1)+x(2).*x(3).*exp(2.*E11_1_50+2.*E22_1_50)))./(2.*E22_1_50+1)) - S22_1_50).^2) ... 
+ sum(((4.*E11_50_1.*(x(1)+x(2).*x(3).*exp(2.*E11_50_1+2.*E22_1_50))+4.*x(4).*E11_50_1.*(2.*E11_50_1+1).*exp(x(5).*((2.*E11_50_1).^2))./(2.*E11_50_1+1)) - S11_50_1).^2 + (((4.*E22_50_1.*(x(1)+x(2).*x(3).*exp(2.*E11_50_1+2.*E22_50_1)))./(2.*E22_50_1+1)) - S22_50_1).^2) ... 

% obj_fun=matlabFunction(sum((sym(fun_11)-S11).^2+(sym(fun_22)-S22).^2));

% Minimization of objective function using fmincon
x0=[1,1,1,1,1]; % initial guesses
lb=[-inf,-inf,2,-inf,-inf]; % lower bound for parameters
ub=[inf,inf,2,inf,inf]; % upper bound for parameters
A=[0,0,0,0,0;0,0,0,0,0;0,0,0,0,0;0,0,0,0,0;0,0,0,0,0]; % Variables to enforce inequality constraint
b=[0,0,0,0,0]; % Variables to enforce inequality constraint
options = optimoptions('fmincon','Display','iter','Algorithm','sqp'); %Options to control execution of fmincon
options = optimoptions('fmincon','Display','iter','Algorithm','sqp','MaxIterations',10000,'MaxFunEval',100000,'StepTolerance',1e-20); % fmincon
x = fmincon(obj_fun,x0,A,b,[],[],lb,ub,[],options);

% Backed out coefficients
c1=x(1)
D1=x(2)
D2=x(3)
k1=x(4)
k2=x(5)
% Matrix form of coeffecients
D=[c1,D1,D2,k1,k2];

% Total R_square
SSres= ... + sum(((4.*E11_1_1.*(x(1)+x(2).*x(3).*exp(2.*E11_1_1+2.*E22_1_1))+4.*x(4).*E11_1_1.*(2.*E11_1_1+1).*exp(x(5)).*(2.*E11_1_1).^2))./(2.*E11_1_1+1)) - S11_1_1).^2+(((4.*E22_1_1.*(x(1)+x(2).*x(3).*exp(2.*E11_1_1+2.*E22_1_1)))./(2.*E22_1_1+1)) - S22_1_1).^2)...
+ sum(((4.*E11_1_75.*(x(1)+x(2).*x(3).*exp(2.*E11_1_75+2.*E22_1_75))+4.*x(4).*E11_1_75.*(2.*E11_1_75+1).*exp(x(5)).*(2.*E11_1_75).^2))./(2.*E11_1_75+1)) - S11_1_75).^2+(((4.*E22_1_75.*(x(1)+x(2).*x(3).*exp(2.*E11_1_75+2.*E22_1_75)))./(2.*E22_1_75+1)) - S22_1_75).^2)...
+ sum(((4.*E11_1_50.*(x(1)+x(2).*x(3).*exp(2.*E11_1_50+2.*E22_1_50))+4.*x(4).*E11_1_50.*(2.*E11_1_50+1).*exp(x(5)).*(2.*E11_1_50).^2))./(2.*E11_1_50+1)) - S11_1_50).^2+(((4.*E22_1_50.*(x(1)+x(2).*x(3).*exp(2.*E11_1_50+2.*E22_1_50)))./(2.*E22_1_50+1)) - S22_1_50).^2)...
+ sum(((4.*E11_50_1.*(x(1)+x(2).*x(3).*exp(2.*E11_50_1+2.*E22_50_1))+4.*x(4).*E11_50_1.*(2.*E11_50_1+1).*exp(x(5)).*(2.*E11_50_1).^2))./(2.*E11_50_1+1)) - S11_50_1).^2+(((4.*E22_50_1.*(x(1)+x(2).*x(3).*exp(2.*E11_50_1+2.*E22_50_1)))./(2.*E22_50_1+1)) - S22_50_1).^2));

c_bar1=mean2(S11_1_1);
 r_bar1=mean2(S22_1_1);
c_bar2=mean2(S11_1_75);
r_bar2=mean2(S22_1_75);
c_bar3=mean2(S11_75_1);
r_bar3=mean2(S22_75_1);
c_bar4=mean2(S11_1_50);
r_bar4=mean2(S22_1_50);
c_bar5=mean2(S11_50_1);
r_bar5=mean2(S22_50_1);

SStot= ...
+ sum(((S11_1_1-c_bar1).^2)+((S22_1_1-r_bar1).^2))...
+ sum(((S11_1_75-c_bar2).^2)+((S22_1_75-r_bar2).^2))...
+ sum(((S11_75_1-c_bar3).^2)+((S22_75_1-r_bar3).^2))...
+ sum(((S11_50_1-c_bar4).^2)+((S22_50_1-r_bar4).^2))...
+ sum(((S11_50_1-c_bar5).^2)+((S22_50_1-r_bar5).^2));

R_square_combine=1-(SSres/SStot)

%Export constants into excel sheet
output=[c1,D1,D2,k1,k2, R_square_combine];
h=['c1', 'D1', 'D2', 'k1', 'k2', 'R_square_combine'];
m=[h,num2cell(output)];
filename='Modified M-R (Anisotropic) Constants.xlsx';
% Generated values for plotting model fit
E11_1_1_m=linspace(0,max(E11_1_1),1000);
E22_1_1_m=linspace(0,max(E22_1_1),1000);
model11_1_1_m=(4.*E11_1_1_m.*(x(1)+x(2).*x(3).*exp(2.*E11_1_1_m+2.*E22_1_1_m) )+4.*x(4).*E11_1_1_m.*(2.*E11_1_1_m+1).*exp(x(5).*x(2).*E11_1_1_m.^2))./(2.*E11_1_1_m+1);
model22_1_1_m=(4.*E22_1_1_m.*(x(1)+x(2).*x(3).*exp(2.*E11_1_1_m+2.*E22_1_1_m) ))/(2.*E22_1_1_m+1);
model11_1_1_m=linspace(0,max(model11_1_1_m),1000);
model22_1_1_m=linspace(0,max(model22_1_1_m),1000);

E11_1_75_m=linspace(0,max(E11_1_75),1000);
E22_1_75_m=linspace(0,max(E22_1_75),1000);
model11_1_75_m=(4.*E11_1_75_m.*(x(1)+x(2).*x(3).*exp(2.*E11_1_75_m+2.*E22_1_75_m) )+4.*x(4).*E11_1_75_m.*(2.*E11_1_75_m+1).*exp(x(5).*x(2).*E11_1_75_m.^2))./(2.*E11_1_75_m+1);
model22_1_75_m=(4.*E22_1_75_m.*(x(1)+x(2).*x(3).*exp(2.*E11_1_75_m+2.*E22_1_75_m) ))/(2.*E22_1_75_m+1);
model11_1_75_m=linspace(0,max(model11_1_75_m),1000);
model22_1_75_m=linspace(0,max(model22_1_75_m),1000);

E11_75_1_m=linspace(0,max(E11_75_1),1000);
E22_75_1_m=linspace(0,max(E22_75_1),1000);
model11_75_1_m=(4.*E11_75_1_m.*(x(1)+x(2).*x(3).*exp(2.*E11_75_1_m+2.*E22_75_1_m) )+4.*x(4).*E11_75_1_m.*(2.*E11_75_1_m+1).*exp(x(5).*x(2).*E11_75_1_m.^2))./(2.*E11_75_1_m+1);
model22_75_1_m=(4.*E22_75_1_m.*(x(1)+x(2).*x(3).*exp(2.*E11_75_1_m+2.*E22_75_1_m) ))/(2.*E22_75_1_m+1);
model11_75_1_m=linspace(0,max(model11_75_1_m),1000);
model22_75_1_m=linspace(0,max(model22_75_1_m),1000);

E11_50_1_m=linspace(0,max(E11_50_1),1000);
E22_50_1_m=linspace(0,max(E22_50_1),1000);
model11_50_1_m=(4.*E11_50_1_m.*(x(1)+x(2).*x(3).*exp(2.*E11_50_1_m+2.*E22_50_1_m) )+4.*x(4).*E11_50_1_m.*(2.*E11_50_1_m+1).*exp(x(5).*x(2).*E11_50_1_m.^2))./(2.*E11_50_1_m+1);
model22_50_1_m=(4.*E22_50_1_m.*(x(1)+x(2).*x(3).*exp(2.*E11_50_1_m+2.*E22_50_1_m) ))/(2.*E22_50_1_m+1);
model11_50_1_m=linspace(0,max(model11_50_1_m),1000);
model22_50_1_m=linspace(0,max(model22_50_1_m),1000);

% Plot:
% Large matrix containing all values
E11=[E11_1_1, E11_1_75, E11_75_1, E11_1_50, E11_50_1];
E22=[E22_1_1, E22_1_75, E22_75_1, E22_1_50, E22_50_1];
S11=[S11_1_1, S11_1_75, S11_75_1, S11_1_50, S11_50_1];
S22=[S22_1_1, S22_1_75, S22_75_1, S22_1_50, S22_50_1];
model11=[model11_1_1_m,model11_1_75_m,model11_75_1_m,model11_1_50_m,model11_50_1_m];
model22=[model22_1_1_m,model22_1_75_m,model22_75_1_m,model22_1_50_m,model22_50_1_m];

% Figure (1) plots Individual ratios of the circumferential and radial
together (5 figures in subplot) Figure is very wide to insure that plots
are displayed properly. Save figure as jpeg or desired file type.

% Size of markers in scatterplot
sz=15;

figure (1)
subplot (1,5,1)
scatter(E11_1_1,S11_1_1,sz,'k'); hold on;
scatter(E22_1_1,S22_1_1,sz,'r'); hold on;
plot(E11_1_1_m,model11_1_1_m,'r',E22_1_1_m,model22_1_1_m,'r'); hold off;
Emax_1_1=max(max(max(E11_1_1)),(max(max(E22_1_1)))); % max E for axis limits
Smax_1_1=max(max(max(S11_1_1)),(max(max(S22_1_1)))); % max S for axis limits
axis([0 Emax_1_1+0.05 0 Smax_1_1+10]); % add 0.1 for max E and 10 for max S
legend('Experimental-Circumferential','S22-Experimental-Radial','Model Fit-Circumferential','Model Fit-Radial')
title('1:1')
xlabel('Green Strain')
ylabel('2nd P-K Stress (kPa)')
set(gca,'Ytick',0:20:Smax_1_1+10);
set(gca,'Xtick',0:0.1:Emax_1_1+0.05);

figure (1)
subplot (1,5,2)
scatter(E11_1_75,S11_1_75,sz,'k'); hold on;
scatter(E22_1_75,S22_1_75,sz,'r'); hold on;
plot(E11_1_75_m,model11_1_75_m,'r',E22_1_75_m,model22_1_75_m,'r'); hold off;
Emax_1_75=max(max(max(E11_1_75)),(max(max(E22_1_75)))); % max E for axis limits
Smax_1_75=max(max(max(S11_1_75)),(max(max(S22_1_75)))); % max S for axis limits
axis([0 Emax_1_75+0.05 0 Smax_1_75+10]); % add 0.1 for max E and 10 for max S
legend('Experimental-Circumferential','S22-Experimental-Radial','Model Fit-Circumferential','Model Fit-Radial')
title('0.75:1')
xlabel('Green Strain')
ylabel('2nd P-K Stress (kPa)')
set(gca,'Ytick',0:20:Smax_1_75+10);
set(gca,'Xtick',0:0.1:Emax_1_75+0.05);
set(findobj(gcf, 'type', 'axes'), 'FontName', 'Arial', 'FontSize', 18, 'FontWeight', 'Bold', 'LineWidth', 2);
box on;

figure (1)
subplot (1,5,3)
sz=15;
scatter(E11_75_1,S11_75_1,sz,'k'); hold on;
scatter(E22_75_1,S22_75_1,sz,'r'); hold on;
plot(E11_75_1_m,model11_75_1_m,'r',E22_75_1_m,model22_75_1_m,'r'); hold off;

Emax_75_1=max(max(max(E11_75_1)),(max(max(E22_75_1)))); % max E for axis limits (x limit)
Smax_75_1=max(max(max(S11_75_1)),(max(max(S22_75_1)))); % max S for axis limits (y limit)
axis([0 Emax_75_1+0.05 0 Smax_75_1+10]) % add 0.1 for max E and 10 for max S
% legend('Experimental-Circumferential','S22-Experimental-Radial','Model Fit-Circumferential','Model Fit-Radial')
% title('1:0.75')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
set(gca, 'Ytick', 0:20:Smax_75_1+10);
set(gca, 'Xtick', 0:0.1:Emax_75_1+0.05);
set(findobj(gcf, 'type', 'axes'), 'FontName', 'Arial', 'FontSize', 18, 'FontWeight', 'Bold', 'LineWidth', 2);
box on;

figure (1)
subplot (1,5,4)
scatter(E11_1_50,S11_1_50,sz,'k'); hold on;
scatter(E22_1_50,S22_1_50,sz,'r'); hold on;
plot(E11_1_50_m,model11_1_50_m,'r',E22_1_50_m,model22_1_50_m,'r'); hold off;

Emax_1_50=max(max(max(E11_1_50)),(max(max(E22_1_50)))); % max E for axis limits (x limit)
Smax_1_50=max(max(max(S11_1_50)),(max(max(S22_1_50)))); % max S for axis limits (y limit)
axis([0 Emax_1_50+0.05 0 Smax_1_50+10]) % add 0.1 for max E and 10 for max S
% legend('Experimental-Circumferential','S22-Experimental-Radial','Model Fit-Circumferential','Model Fit-Radial')
% title('0.5:1')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
set(gca, 'Ytick', 0:20:Smax_1_50+10);
set(gca, 'Xtick', 0:0.1:Emax_1_50+0.05);
set(findobj(gcf, 'type', 'axes'), 'FontName', 'Arial', 'FontSize', 18, 'FontWeight', 'Bold', 'LineWidth', 2);
box on;

figure (1)
subplot (1,5,5)
scatter(E11_50_1,S11_50_1,sz,'k'); hold on;
scatter(E22_50_1,S22_50_1,sz,'r'); hold on;
plot(E11_50_1_m,model11_50_1_m,'r',E22_50_1_m,model22_50_1_m,'r'); hold off;
Emax_50_1=max(max(max(E11_50_1)),(max(max(E22_50_1)))); % max E for axis limits (x limit)
Smax_50_1=max(max(max(S11_50_1)),(max(max(S22_50_1)))); % max S for axis limits (y limit)
axis([0 Emax_50_1+0.05 0 Smax_50_1+10]) % add 0.1 for max E and 10 for max S
% legend('Experimental-Circumferential','S22-Experimental-Radial','Model Fit-Circumferential','Model Fit-Radial')
% title('1:0.5')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
set(gca,'Ytick', 0:20:Smax_50_1+10);
set(gca,'Xtick', 0:0.1:Emax_50_1+0.05);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold','LineWidth',2);
box on;

% Figure (2) plots all ratios with circumferential and radial separate
% (2 figures in subplot). Save figure as jpeg or desired file type.

x0=10;
y0=10;
width=1600;
height=200;
set(gcf,'units','points','position',[x0,y0,width,height])

% Note: label of each ratio 1:1=circle; 1:0.75=astrix
figure (2)
subplot (1,2,1)
scatter(E11_1_1,S11_1_1,sz,'k'); hold on;
scatter(E11_1_75,S11_1_75,sz,'r'); hold on;
scatter(E11_1_75,S11_1_75,sz,'g','v','filled','MarkerEdgeColor',[0 0 0],'LineWidth',0.6); hold on;
scatter(E11_1_50,S11_1_50,sz,'y','^','filled','MarkerEdgeColor',[0 0 0],'LineWidth',0.6); hold on;
scatter(E11_50_1,S11_50_1,sz,'b','s','filled','MarkerEdgeColor',[0 0 0],'LineWidth',0.6); hold on;
plot(E11_1_1_m,model11_1_1_m,'r',E11_1_75_m,model11_1_75_m,'r',E11_1_50_m,model11_1_50_m,'r',E11_50_1_m,model11_50_1_m,'r'); hold off;

Emax=max(max(max(E11)),(max(max(E22)))); % max E for axis limits (x limit)
Smax=max(max(max(S11)),(max(max(S22)))); % max S for axis limits (y limit)
Emax=max(max(E11)); % max E for axis limits (x limit)
Smax=max(max(max(S11)),(max(model11))); % max S for axis limits (x limit)
axis([0 Emax+0.05 0 Smax+10]); % add 0.05 for max E and 10 for max S
% legend('Experimental','Experimental 1:0.75','Experimental 0.75:1','Experimental 1:0.5','Experimental 0.5:1','Model Fit 1:1','Model Fit 1:0.75','Model Fit 0.75:1','Model Fit 1:0.5','Model Fit 0.5:1')
% title('Circumferential')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
set(gca,'Ytick', 0:20:Smax+10);
set(gca,'Xtick', 0:0.1:Emax+0.05);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold','LineWidth',2);
box on;
figure (2)
subplot (1,2,2)
scatter(E22_1_1,S22_1_1,sz,'k'); hold on;
scatter(E22_1_75,S22_1_75,sz,'r'); hold on;
scatter(E22_75_1,S22_75_1,sz,'g','v','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;
scatter(E22_1_50,S22_1_50,sz,'y','^','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;
scatter(E22_50_1,S22_50_1,sz,'b','s','filled','MarkerEdgeColor',[0 0 0], 'LineWidth',0.6); hold on;
plot(E22_1_1_m,model22_1_1_m,'r',E22_1_75_m,model22_1_75_m,'r',E22_75_1_m,model22_75_1_m,'r',E22_1_50_m,model22_1_50_m,'r',E22_50_1_m,model22_50_1_m,'r'); hold off;
Emax=max(max(E22)); %max E for axis limits (x limit)
Smax=(max(max(max(S22)),(max(model22)))); %max S for axis limits (y limit)
axis([0 Emax+0.05 0 Smax+10]); % add 0.1 for max E and 10 for max S
% legend('Experimental 1:1','Experimental 1:0.75','Experimental 0.75:1','Experimental 1:0.5','Experimental 0.5:1','Model Fit 1:1','Model Fit 1:0.75','Model Fit 0.75:1','Model Fit 1:0.5','Model Fit 0.5:1')
% title('Radial')
% xlabel('Green Strain!')
% ylabel('2nd P-K Stress (kPa)')
set(findobj(gca,'type','axes'),'FontName','Arial','FontSize',12,'FontWeight','Bold', 'LineWidth', 2);
box on;

x0=10;
y0=10;
width=800;
height=250;
set(gcf, 'units','points', 'position',[x0,y0,width,height])
clc
clear all
close all
warning off;

%%Edited by Jessica Perez
%%Version November 26, 2018
%%Written by Alex Khang, October 31, 2018

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%Department of Biomedical Engineering
%Mechanobiology and Soft Materials Laboratory (MSML)
%PI: Dr. Kartik Balachandran

%%This code calculates the constants for the fung-type computational model
%%and graphs the experimental data and model data.

%%Fung-type Model:
%% x=E11, y=E22
%% S11=(c./2).*(exp(c1.*x.^2+c2.*y.^2+2.*c3.*x.*y)).*(2.*c1.*x+2.*c3.*y)
%% S22=(c./2).*(exp(c1.*x.^2+c2.*y.^2+2.*c3.*x.*y)).*(2.*c1.*x+2.*c3.*y)

%Note:
% To extract the files the data should be as follows on the file with
% all the ratio data.
% 1:1: Column A=E11; Column B=E22; Column C=S11; Column D=S22
% 1:0.75: Column I=E11; Column J=E22; Column K=S11; Column L=S22
% 0.75:1: Column Q=E11; Column R=E22; Column S=S11; Column T=S22
% 1:0.5: Column Y=E11; Column Z=E22; Column AA=S11; Column AB=S22
% 0.5:1: Column AG=E11; Column AH=E22; Column AI=S11; Column AJ=S22

%Input:
% File containing ratio data

%Output:
% Excel file:Fung-type Constants.xlsx
% Contains: c,c1,c2,c3,condition data, r square combined, anisotropy
% index
% Graphs that need to be saved as jpeg or other filetype:
% (1) Individual ratios of the circumferential and radial together (5
% figures in subplot)
% Legend:
% Experimental Circumferential=black circles
% Experimental Radial=red circles
% Model Fit=Red Line
% Order:
% left to right= 1:1, 1:0.75, 0.75:1, 1:0.5, 0.5:1
% (2) All ratios with circumferential and radial separate (2 figures in
% subplot)
% Legend:
% Experimental 1:1=black circles
% Experimental 1:0.75=red circles
% Experimental 0.75:1=upside down green triangle
% Experimental 1:0.50 = yellow triangle
% Experimental 0.5:1 = blue square
% Model Fit = Red Line
% Order:
% left to right = Circumferential, Radial
% (3) Contour plots of the Fung-type model

% Note:
% (1) Figures are the size that allows the subplots to be saved properly and not be stretched out.
% (2) Figures have no titles, x and y labels, or legends. I put those when I'm compiling figures in a powerpoint. However, the code to do so is commented so you can just uncomment them for whatever you want on the graph.

% Extracting all ratio data from excel file
[filename, pathname] = uigetfile('*.txt', 'Select file');
data = xlsread(filename);
index = size(data, 1);

% Seperating data by ratios 1:1, 1:0.75, 0.75:1, 1:0.5, 0.5:1
E11_1_1 = data([1:index], 1);
E22_1_1 = data([1:index], 2);
S11_1_1 = data([1:index], 3);
S22_1_1 = data([1:index], 4);

E11_1_75 = data([1:index], 9);
E22_1_75 = data([1:index], 10);
S11_1_75 = data([1:index], 11);
S22_1_75 = data([1:index], 12);

E11_75_1 = data([1:index], 17);
E22_75_1 = data([1:index], 18);
S11_75_1 = data([1:index], 19);
S22_75_1 = data([1:index], 20);

E11_1_50 = data([1:index], 25);
E22_1_50 = data([1:index], 26);
S11_1_50 = data([1:index], 27);
S22_1_50 = data([1:index], 28);

E11_50_1 = data([1:index], 33);
E22_50_1 = data([1:index], 34);
S11_50_1 = data([1:index], 35);
S22_50_1 = data([1:index], 36);

% Large matrix containing all values
E11 = [E11_1_1, E11_1_75, E11_75_1, E11_1_50, E11_50_1];
E22 = [E22_1_1, E22_1_75, E22_75_1, E22_1_50, E22_50_1];
S11 = [S11_1_1, S11_1_75, S11_75_1, S11_1_50, S11_50_1];
S22 = [S22_1_1, S22_1_75, S22_75_1, S22_1_50, S22_50_1];

% % Model formulation
% syms c a1 a2 a3 Exx Eyy real
% Q = a1*Exx^2 + a2*Eyy^2 + 2*a3*Exx*Eyy;
% W=0.5*c*(exp(Q)-1); % fun_11=matlabFunction(diff(W,Exx)); %symbolic % fun_22=matlabFunction(diff(W,Eyy)); %symbolic % clear Exx Eyy

% Objective function. This is the sum of squares of the residuals. I had to % write out the complete equations because I'm not sure yet how to % incorporate function handles fun_11 and fun_22 into this.

obj_fun = @(x) ...
+ sum(((x(1).*exp(E11_1_1.^2.*x(2)+E22_1_1.^2.*x(3)+E11_1_1.*E22_1_1.*x(4).*2.0)\n  .*(E11_1_1.*x(2).*2.0+E22_1_1.*x(4).*2.0).*1.0./2.0))-S11_1_1).^2\n  + ((x(1).*exp(E11_1_1.^2.*x(2)+E22_1_1.^2.*x(3)+E11_1_1.*E22_1_1.*x(4).*2.0)\n  .*(E11_1_1.*x(4).*2.0+E22_1_1.*x(3).*2.0).*1.0./2.0))-S22_1_1).^2\n  + ((x(1).*exp(E11_1_75.^2.*x(2)+E22_1_75.^2.*x(3)+E11_1_75.*E22_1_75.*x(4).*2.0)\n  .*(E11_1_75.*x(2).*2.0+E22_1_75.*x(4).*2.0).*1.0./2.0))-S11_75_1).^2\n  + ((x(1).*exp(E11_1_75.^2.*x(2)+E22_1_75.^2.*x(3)+E11_1_75.*E22_1_75.*x(4).*2.0)\n  .*(E11_1_75.*x(4).*2.0+E22_1_75.*x(3).*2.0).*1.0./2.0))-S22_75_1).^2\n  + ((x(1).*exp(E11_1_50.^2.*x(2)+E22_1_50.^2.*x(3)+E11_1_50.*E22_1_50.*x(4).*2.0)\n  .*(E11_1_50.*x(2).*2.0+E22_1_50.*x(4).*2.0).*1.0./2.0))-S11_50_1).^2\n  + ((x(1).*exp(E11_1_50.^2.*x(2)+E22_1_50.^2.*x(3)+E11_1_50.*E22_1_50.*x(4).*2.0)\n  .*(E11_1_50.*x(4).*2.0+E22_1_50.*x(3).*2.0).*1.0./2.0))-S22_50_1).^2\n  + ((x(1).*exp(E11_50_1.^2.*x(2)+E22_50_1.^2.*x(3)+E11_50_1.*E22_50_1.*x(4).*2.0)\n  .*(E11_50_1.*x(2).*2.0+E22_50_1.*x(4).*2.0).*1.0./2.0))-S11_50_1).^2\n  + ((x(1).*exp(E11_50_1.^2.*x(2)+E22_50_1.^2.*x(3)+E11_50_1.*E22_50_1.*x(4).*2.0)\n  .*(E11_50_1.*x(4).*2.0+E22_50_1.*x(3).*2.0).*1.0./2.0))-S22_50_1).^2);

% obj_fun=matlabFunction(sum((sym(fun_11)-S11).^2+(sym(fun_22)-S22).^2));

% Minimization of objective function using fmincon
x0=[1,4,1,1]; % initial guesses
lb=[0,-inf,-inf,-inf]; % lower bound for parameters
ub=[inf,inf,inf,inf]; % upper bound for parameters
A=[0,-1,0,1,0,-1,1]; % Variables to enforce inequality constraint A1>A3
b=[0,0]; % Variables to enforce inequality constraint A2>A3
options = optimoptions('fmincon','Display','iter','Algorithm','sqp');
%Options to control execution of fmincon
% options = optimoptions('fmincon','Display','iter','Algorithm','sgp','MaxIterations',10000,'MaxFunEval',100000,'StepTolerance',1e-20);
x = fmincon(obj_fun,x0,A,b,[],[],lb,ub,[],options);
% Backed out coefficients
c = x(1)
c1 = x(2)
c2 = x(3)
c3 = x(4)

% Matrix form of coefficients
D = [c, c1; c2, c3];

% Condition number. This must be less than or equal to 200 for acceptable
% numerical convergence.
Condition_number = norm(D, 1) * norm(inv(D), 1)

% Total R_square
SSres = ...
  + sum(((x(1) .* exp(E11_1_1.^2.*x(2)+E22_1_1.^2.*x(3)+E11_1_1.*E22_1_1.*x(4).*2.0).*(E11_1_1.*x(2).*2.0+E22_1_1.*x(4).*2.0).*1.0./2.0)) - S11_1_1).^2 + ((x(1) .* exp(E11_1_1.^2.*x(2)+E22_1_1.^2.*x(3)+E11_1_1.*E22_1_1.*x(4).*2.0).*(E11_1_1.*x(4).*2.0+E22_1_1.*x(3).*2.0).*1.0./2.0)) - S22_1_1).^2)
  ...
  + sum(((x(1) .* exp(E11_1_75.^2.*x(2)+E22_1_75.^2.*x(3)+E11_1_75.*E22_1_75.*x(4).*2.0).*(E11_1_75.*x(2).*2.0+E22_1_75.*x(4).*2.0).*1.0./2.0)) - S11_1_75).^2 + ((x(1) .* exp(E11_1_75.^2.*x(2)+E22_1_75.^2.*x(3)+E11_1_75.*E22_1_75.*x(4).*2.0).*(E11_1_75.*x(4).*2.0+E22_1_75.*x(3).*2.0).*1.0./2.0)) - S22_1_75).^2)
  ...
  + sum(((x(1) .* exp(E11_50_1.^2.*x(2)+E22_50_1.^2.*x(3)+E11_50_1.*E22_50_1.*x(4).*2.0).*(E11_50_1.*x(2).*2.0+E22_50_1.*x(4).*2.0).*1.0./2.0)) - S11_50_1).^2 + ((x(1) .* exp(E11_50_1.^2.*x(2)+E22_50_1.^2.*x(3)+E11_50_1.*E22_50_1.*x(4).*2.0).*(E11_50_1.*x(4).*2.0+E22_50_1.*x(3).*2.0).*1.0./2.0)) - S22_50_1).^2);

  c_bar1 = mean2(S11_1_1);
c_bar2 = mean2(S11_1_75);
c_bar3 = mean2(S11_1_50);
c_bar4 = mean2(S11_50_1);
c_bar5 = mean2(S11_50_1);
r_bar1 = mean2(S22_1_1);
r_bar2 = mean2(S22_1_75);
r_bar3 = mean2(S22_1_50);
r_bar4 = mean2(S22_50_1);
r_bar5 = mean2(S22_50_1);
SStot= ...
+ sum(((S11_1_1-c_bar1).^2)+((S22_1_1-r_bar1).^2))...
+ sum(((S11_1_75-c_bar2).^2)+((S22_1_75-r_bar2).^2))...
+ sum(((S11_75_1-c_bar3).^2)+((S22_75_1-r_bar3).^2))...
+ sum(((S11_1_50-c_bar4).^2)+((S22_1_50-r_bar4).^2))...
+ sum(((S11_50_1-c_bar5).^2)+((S22_50_1-r_bar5).^2));

R_square_combine=1-(SSres/SStot)

%Anisotropy Index
AI=min((c1+c3)./(c2+c3),(c2+c3)./(c1+c3))

%Export constants into excel sheet
output=[c, c1, c2, c3, Condition_number, R_square_combine, AI];
h={'c', 'c1', 'c2', 'c3', 'Condition_number', 'R_square_combine', 'AI'};
m=[h;num2cell(output)];
filename='Fung-type Constants.xlsx';
xlswrite(filename,m);

% Generated values for plotting model fit
E11_1_1_m=linspace(0,max(E11_1_1),1000);
E22_1_1_m=linspace(0,max(E22_1_1),1000);
model11_1_1_m=x(1).*exp(E11_1_1_m.^2.*x(2)+E22_1_1_m.^2.*x(3)+E11_1_1_m.*E22_1_1_m.*x(4).*2.0).*(E11_1_1_m.*x(2).*2.0+E22_1_1_m.*x(4).*2.0).*(1.0./2.0);
model22_1_1_m=x(1).*exp(E11_1_1_m.^2.*x(2)+E22_1_1_m.^2.*x(3)+E11_1_1_m.*E22_1_1_m.*x(4).*2.0).*(E11_1_1_m.*x(4).*2.0+E22_1_1_m.*x(3).*2.0).*(1.0./2.0);

E11_1_75_m=linspace(0,max(E11_1_75),1000);
E22_1_75_m=linspace(0,max(E22_1_75),1000);
model11_1_75_m=x(1).*exp(E11_1_75_m.^2.*x(2)+E22_1_75_m.^2.*x(3)+E11_1_75_m.*E22_1_75_m.*x(4).*2.0).*(E11_1_75_m.*x(2).*2.0+E22_1_75_m.*x(4).*2.0).*(1.0./2.0);
model22_1_75_m=x(1).*exp(E11_1_75_m.^2.*x(2)+E22_1_75_m.^2.*x(3)+E11_1_75_m.*E22_1_75_m.*x(4).*2.0).*(E11_1_75_m.*x(4).*2.0+E22_1_75_m.*x(3).*2.0).*(1.0./2.0);

E11_75_1_m=linspace(0,max(E11_75_1),1000);
E22_75_1_m=linspace(0,max(E22_75_1),1000);
model11_75_1_m=x(1).*exp(E11_75_1_m.^2.*x(2)+E22_75_1_m.^2.*x(3)+E11_75_1_m.*E22_75_1_m.*x(4).*2.0).*(E11_75_1_m.*x(2).*2.0+E22_75_1_m.*x(4).*2.0).*(1.0./2.0);
model22_75_1_m=x(1).*exp(E11_75_1_m.^2.*x(2)+E22_75_1_m.^2.*x(3)+E11_75_1_m.*E22_75_1_m.*x(4).*2.0).*(E11_75_1_m.*x(4).*2.0+E22_75_1_m.*x(3).*2.0).*(1.0./2.0);

E11_1_50_m=linspace(0,max(E11_1_50),1000);
E22_1_50_m=linspace(0,max(E22_1_50),1000);
model11_1_50_m=x(1).*exp(E11_1_50_m.^2.*x(2)+E22_1_50_m.^2.*x(3)+E11_1_50_m.*E22_1_50_m.*x(4).*2.0).*(E11_1_50_m.*x(2).*2.0+E22_1_50_m.*x(4).*2.0).*(1.0./2.0);
model22_1_50_m=x(1).*exp(E11_1_50_m.^2.*x(2)+E22_1_50_m.^2.*x(3)+E11_1_50_m.*E22_1_50_m.*x(4).*2.0).*(E11_1_50_m.*x(4).*2.0+E22_1_50_m.*x(3).*2.0).*(1.0./2.0);

E11_50_1_m=linspace(0,max(E11_50_1),1000);
E22_50_1_m=linspace(0,max(E22_50_1),1000);  % E22_50_1 is the 2nd P-K Stress (kPa)
model11_50_1_m=x(1).*exp(E11_50_1_m.^2.*x(2)+E22_50_1_m.^2.*x(3)+E11_50_1_m.*
                  E22_50_1_m.*x(4).*2.0).*E11_50_1_m.*x(2)*2.0+2.*E22_50_1_m.*x(4)*2.0.*
                  (1.0./2.0);
model22_50_1_m=x(1).*exp(E11_50_1_m.^2.*x(2)+E22_50_1_m.^2.*x(3)+E11_50_1_m.*
                  E22_50_1_m.*x(4).*2.0).*E11_50_1_m.*x(4)*2.0+2.*E22_50_1_m.*x(3)*2.0.*
                  (1.0./2.0);

%Plot fit lines:

%Figure (1) plots Individual ratios of the circumferential and radial %together (5 figures in subplot)Figure is very wide to insure that plots %are displayed properly. Save figure as jpeg or desired file type.

%Size of markers in scatterplot  
sz=15;

figure (1)  
subplot (1,5,1)  
scatter(E11_1_1,S11_1_1,sz,'k'); hold on;  
scatter(E22_1_1,S22_1_1,sz,'r'); hold on;  
plot(E11_1_1_m,model11_1_1_m,'r',E22_1_1_m,model22_1_1_m,'r'); hold off;  
% legend('Experimental-Circumferential','Model Fit-Circumferential','Model Fit-Radial')  
% title('1:1')  
% xlabel('Green Strain')  
% ylabel('2nd P-K Stress (kPa)')  
Emax=max(max(max(E11_1_1)),(max(max(E22_1_1)))); %max E for axis limits (x limit)  
Smax=max(max(max(S11_1_1)),(max(max(S22_1_1),max(max(model11_1_1_m),max(max(model22_1_1_m)))))); %max S for axis limits (y limit)  
axis([0 Emax+0.05 0 Smax+10]); % add 0.1 for max E and 10 for max S  
set(gca,'Ytick',0:20:Smax+10);  
set(gca,'Xtick',0:0.1:Emax+0.05);  
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold', 'LineWidth',2);  
box on;

figure (1)  
subplot (1,5,2)  
scatter(E11_1_75,S11_1_75,sz,'k'); hold on;  
scatter(E22_1_75,S22_1_75,sz,'r'); hold on;  
plot(E11_1_75_m,model11_1_75_m,'r',E22_1_75_m,model22_1_75_m,'r'); hold off;  
% legend('Experimental-Circumferential','Model Fit-Circumferential','Model Fit-Radial')  
% title('0.75:1')  
% xlabel('Green Strain')  
% ylabel('2nd P-K Stress (kPa)')  
Emax=max(max(max(E11_1_75)),(max(max(E22_1_75)))); %max E for axis limits (x limit)  
Smax=max(max(max(S11_1_75)),(max(max(S22_1_75),max(max(model11_1_75_m),max(max(model22_1_75_m)))))); %max S for axis limits (y limit)  
axis([0 Emax+0.05 0 Smax+10]); % add 0.1 for max E and 10 for max S  
set(gca,'Ytick',0:20:Smax+10);  
set(gca,'Xtick',0:0.1:Emax+0.05);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold', 'LineWidth', 2); box on;

figure (1)
subplot (1,5,3)
scatter(E11_75_1,S11_75_1,sz,'k'); hold on;
scatter(E22_75_1,S22_75_1,sz,'r'); hold on;
plot(E11_75_1_m,model11_75_1_m,'r',E22_75_1_m,model22_75_1_m,'r'); hold off;
% legend('Experimental-Circumferential','S22-Experimental-Radial','Model Fit-Circumferential','Model Fit-Radial')
% title('1:0.75')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
Emax=max(max(max(E11_75_1)),(max(max(E22_75_1)))); %max E for axis limits (x limit)
Smax=max(max(max(S11_75_1)),(max(max(S22_75_1),max(model11_75_1_m),max(model22_75_1_m)))); %max S for axis limits (y limit)
axis([0 Emax+0.05 0 Smax+10]); % add 0.1 for max E and 10 for max S
set(gca,'Ytick', 0:20:Smax+10);
set(gca,'Xtick', 0:0.1:Emax+0.05);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold', 'LineWidth', 2); box on;

figure (1)
subplot (1,5,4)
scatter(E11_1_50,S11_1_50,sz,'k'); hold on;
scatter(E22_1_50,S22_1_50,sz,'r'); hold on;
plot(E11_1_50_m,model11_1_50_m,'r',E22_1_50_m,model22_1_50_m,'r'); hold off;
% legend('Experimental-Circumferential','S22-Experimental-Radial','Model Fit-Circumferential','Model Fit-Radial')
% title('0.5:1')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
Emax=max(max(max(E11_1_50)),(max(max(E22_1_50)))); %max E for axis limits (x limit)
Smax=max(max(max(S11_1_50)),(max(max(S22_1_50),max(model11_1_50_m),max(model22_1_50_m)))); %max S for axis limits (y limit)
axis([0 Emax+0.05 0 Smax+10]); % add 0.1 for max E and 10 for max S
set(gca,'Ytick', 0:20:Smax+10);
set(gca,'Xtick', 0:0.1:Emax+0.05);
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold', 'LineWidth', 2); box on;

figure (1)
subplot (1,5,5)
scatter(E11_50_1,S11_50_1,sz,'k'); hold on;
scatter(E22_50_1,S22_50_1,sz,'r'); hold on;
plot(E11_50_1_m,model11_50_1_m,'r',E22_50_1_m,model22_50_1_m,'r'); hold off;
% legend('Experimental-Circumferential','S22-Experimental-Radial','Model Fit-Circumferential','Model Fit-Radial')
% title('1:0.5')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
E_{max} = \max(\max(\max(E_{11_50_1})), (\max(\max(E_{22_50_1})))) \quad \% \text{max E for axis limits (x limit)}

S_{max} = \max(\max(\max(S_{11_50_1})), (\max(\max(S_{22_50_1})), (\max(\max(\text{model}_{11_50_1_m})), (\max(\max(\text{model}_{22_50_1_m})))))) \quad \% \text{max S for axis limits (y limit)}

\text{axis}([0 \ E_{max}+0.05 \ 0 \ S_{max}+10]); \quad \% \text{add 0.1 for max E and 10 for max S}

\text{set(gca,'Ytick', 0:20:S_{max}+10)};

\text{set(gca,'Xtick', 0:0.1:E_{max}+0.05)};

\text{set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold','LineWidth',2);}

\text{box on};

x0=10;
y0=10;
width=1800;
height=200;
\text{set(gcf,'units','points','position',[x0,y0,width,height])}

\% Figure (2) plots all ratios with circumferential and radial separate
\% (2 figures in subplot). Save figure as jpeg or desired file type.

\% Large matrix containing all values
E_{11} = [E_{11_1_1}, E_{11_1_75}, E_{11_75_1}, E_{11_1_50}, E_{11_50_1}];
E_{22} = [E_{22_1_1}, E_{22_1_75}, E_{22_75_1}, E_{22_1_50}, E_{22_50_1}];
S_{11} = [S_{11_1_1}, S_{11_1_75}, S_{11_75_1}, S_{11_1_50}, S_{11_50_1}];
S_{22} = [S_{22_1_1}, S_{22_1_75}, S_{22_75_1}, S_{22_1_50}, S_{22_50_1}];
\text{model}_{xx} = [\text{model}_{11_1_1_m}, \text{model}_{11_1_75_m}, \text{model}_{11_75_1_m}, \text{model}_{11_1_50_m}, \text{model}_{11_50_1_m}];
\text{model}_{yy} = [\text{model}_{22_1_1_m}, \text{model}_{22_1_75_m}, \text{model}_{22_75_1_m}, \text{model}_{22_1_50_m}, \text{model}_{22_50_1_m}];

\% Note: label of each ratio-1:1=circle; 1:0.75=astrix

\text{figure (2)}
\text{subplot (1,2,1)}
\text{scatter(E_{11_1_1},S_{11_1_1},sz,'k'); hold on;}
\text{scatter(E_{11_1_75},S_{11_1_75},sz,'r'); hold on;}
\text{scatter(E_{11_75_1},S_{11_75_1},sz,'g','v','filled','MarkerEdgeColor',[0 0 0],'LineWidth',0.6); hold on;}
\text{scatter(E_{11_1_50},S_{11_1_50},sz,'y','^','filled','MarkerEdgeColor',[0 0 0],'LineWidth',0.6); hold on;}
\text{scatter(E_{11_50_1},S_{11_50_1},sz,'b','s','filled','MarkerEdgeColor',[0 0 0],'LineWidth',0.6); hold on;}
\text{plot(E_{11_1_1_m},\text{model}_{11_1_1_m},'r',E_{11_1_75_m},\text{model}_{11_1_75_m},'r',E_{11_75_1_m},\text{model}_{11_75_1_m},'r',E_{11_1_50_m},\text{model}_{11_1_50_m},'r',E_{11_50_1_m},\text{model}_{11_50_1_m},'r'); hold off;}
\% legend('Experimental 1:1','Experimental 1:0.75','Experimental 0.75:1','Experimental 0.5:1','Model Fit 1:1','Model Fit 0.75:1','Model Fit 0.5:1')
\% title('Circumferential')
\% xlabel('Green Strain')
\% ylabel('2nd P-K Stress (kPa)')
E_{max} = \max(\max(E_{11})); \quad \% \text{max E for axis limits (x limit)}
S_{max} = \max(\max(\max(S_{11})), (\max(\max(\text{model}_{xx})))) \quad \% \text{max S for axis limits (y limit)}
\text{axis([0 E_{max}+0.05 0 S_{max}+10]);} \quad \% \text{add 0.1 for max E and 10 for max S}
\text{set(gca,'Ytick', 0:20:S_{max}+10)};
\text{set(gca,'Xtick', 0:0.1:E_{max}+0.05)};
```matlab
set(findobj(gcf, 'type', 'axes'), 'FontName', 'Arial', 'FontSize', 18, 'FontWeight', 'Bold', 'LineWidth', 2);
box on;

figure (2)
subplot (1,2,2)
scatter(E22_1_1,S22_1_1,sz,'k'); hold on;
scatter(E22_1_75,S22_1_75,sz,'r'); hold on;
scatter(E22_75_1,S22_75_1,sz,'g', 'v', 'filled', 'MarkerEdgeColor', [0 0 0], 'LineWidth', 0.6); hold on;
scatter(E22_1_50,S22_1_50,sz,'y', '^', 'filled', 'MarkerEdgeColor', [0 0 0], 'LineWidth', 0.6); hold on;
scatter(E22_50_1,S22_50_1,sz,'b', 's', 'filled', 'MarkerEdgeColor', [0 0 0], 'LineWidth', 0.6); hold on;
plot(E22_1_1_m,model22_1_1_m, 'r', E22_1_75_m,model22_1_75_m, 'r', E22_75_1_m,model22_75_1_m, 'r', E22_1_50_m,model22_1_50_m, 'r', E22_50_1_m,model22_50_1_m, 'r'); hold off;
% legend('Experimental 1:1','Experimental 1:0.75','Experimental 0.75:1','Experimental 1:0.5','Experimental 0.5:1','Model Fit 1:1','Model Fit 1:0.75','Model Fit 0.75:1','Model Fit 1:0.5','Model Fit 0.5:1')
% title('Radial')
% xlabel('Green Strain')
% ylabel('2nd P-K Stress (kPa)')
Emax=max(max(E22)); %max E for axis limits (x limit)
Smax=(max(max(max(S22)), (max(modelyy)))); %max S for axis limits (y limit)
axis([0 Emax+0.05 0 Smax+10]); % add 0.1 for max E and 10 for max S
set(gca, 'Ytick', 0:20:Smax+10);
set(gca, 'Xtick', 0:0.1:Emax+0.05);
set(findobj(gcf, 'type', 'axes'), 'FontName', 'Arial', 'FontSize', 18, 'FontWeight', 'Bold', 'LineWidth', 2);
box on;

x0=10;
y0=10;
width=800;
height=250;
set(gcf, 'units', 'points', 'position', [x0 y0 width height])

%Strain Energy Contour Plot:
%E for circ and rad. Note: There aren't the experimental E but a range to %see how the constants fit
E11cp=[-1.5:0.02:1.5]; %max E for axis limits (x limit)
E22cp=[-1.5:0.02:1.5];

%Strain Energy Equation for Fung-type model
Q=c1.*E11cp.^2+c2.*E22cp.^2+2.*c3.*E11cp.*E22cp;
W=0.5.*c.*(exp(Q)-1);

%Figure of contour plot
figure (3)
[xx,yy]=meshgrid(E11cp,E22cp);
Q=c1.*xx.^2+c2.*yy.^2+2.*c3.*xx.*yy;
W=0.5.*c.*(exp(Q)-1);
contour(xx,yy,W,[0.01 0.5 3 10 30], 'k', 'ShowText', 'on', 'LineWidth', 1.5)
```
set(findobj(gcf,'type','axes'),'FontName','Arial','FontSize',18,'FontWeight','Bold', 'LineWidth', 2, 'XTick',-1.5:0.5:1.5, 'YTick',-1.5:0.5:1.5); 
box on;
% Picrosirius Red Analysis
%
% This program reads in picrosirius red image files and calculates
% percentage of red, orange, yellow and green fibers in the
% image.
%
% INSTRUCTIONS: Select PSR image to analyze. Create a polygon surrounding.
% the region of interest then right click and choose create mask. Copy the
% matrix percentage_royg to excel sheet. Matrix numbers correspond to the
% red, orange, yellow, and green percentages respectively.
%
% INPUT: PSR image
%
% RETURNS: Matrix with red, orange, yellow, and green percentages.
%
% Created by Dr. Kartik Balchandran
% Mechanobiology and Soft Materials Laboratory
% University of Arkansas, Fayetteville, AR 72701
%
% Edited by Jessica Perez January 2017
% Mechanobiology and Soft Materials Laboratory
% University of Arkansas, Fayetteville, AR 72701
%
% Edited by Ishita tandon November 2018
% Mechanobiology and Soft Materials Laboratory
% University of Arkansas, Fayetteville, AR 72701
% Ranges of hue bins updated.
% Hue Values adapted from -
%
clc;
clear all;
close all;

display('Select image');
[filename pathname]=uigetfile('*.*','Select File...');
stain=imread(filename); %Image file name

% select region of interest by tracing polygon and then mask by right
% clicking and selecting creat mask
display('Select region of interest then double click to create mask');
Mask=roipoly(stain);

% RGB channels of image
red = double(stain(:,:,1)); % Red channel
green = double(stain(:,:,2)); % Green channel
blue = double(stain(:,:,3)); % Blue channel

% Mask of RGB channels
Ir=Mask.*red;
Ig=Mask.*green;
Ib=Mask.*blue;
% Recombining RGB channels and changing it from double to uint8
stain_cropped=uint8(cat(3,Ir, Ig, Ib));

figure (1);
subplot(3,3,1);
imshow(stain);
title('Original Image');
subplot(3,3,2);
imshow(stain_cropped)
title('Crop ped Image');

% Magnifies image to simplify the selection of area of intest
stain_cropped=double(stain_cropped);
stain_cropped_norm = stain_cropped./255;
stain_cropped_thres= stain_cropped_norm > 0.1;
stain_cropped_thres=double(stain_cropped_thres);
stain_hsv=rgb2hsv(stain1);
hue_mat=stain_hsv(:,:,1); %h(:,:,1); s(:,:,2); v(:,:,3)
hue_mat1= hue_mat .* 360; % hsv is a 360 degree circle
[height, width]=size(hue_mat1);

% Defines the hue values for a particular color
H1= double(hue_mat1 > 0 & hue_mat1 < 15) + double(hue_mat1 > 345 & hue_mat1 < 360); %red
H2= double(hue_mat1 > 16 & hue_mat1 < 38); %orange
H3= double(hue_mat1 > 39 & hue_mat1 < 63); %yellow
H4= double(hue_mat1 > 64 & hue_mat1 < 150); %green

% Shows the individual red, orange, yellow, green images based on the above mentioned criteria
figure(1)
subplot(3,3,3);
imshow(stain_cropped)
title('HSV Image');
subplot(3,3,4);
imshow(H1)
title('Red pixels');
subplot(3,3,5)
imshow (H2)
title('Orange pixels');
subplot(3,3,7)
imshow (H3)
title('Yellow pixels');
subplot(3,3,8)
imshow (H4)
title('Green pixels')

num_red=0;
num_orange=0;
num_yellow=0;
num_green=0;
num_total_pixels = height .* width;
for i=1:height
    for j=1:width
        if(hue_mat1(i,j) > 0) && (hue_mat1(i,j) < 15) % red pixels
            num_red = num_red + 1;
        elseif(hue_mat1(i,j) > 345) && (hue_mat1(i,j) < 360) % red pixels
            num_red = num_red + 1;
        elseif(hue_mat1(i,j) > 16) && (hue_mat1(i,j) < 38) % orange pixels
            num_orange = num_orange + 1;
        elseif(hue_mat1(i,j) > 39) && (hue_mat1(i,j) < 63) % yellow pixels
            num_yellow = num_yellow + 1;
        elseif(hue_mat1(i,j) > 64) && (hue_mat1(i,j) < 150) % green pixels
            num_green = num_green + 1;
        end
    end
end

% Displays the percentage of fibers that are red, orange, yellow, or green
total = num_red+num_orange+num_yellow+num_green;
red = (num_red./total).*100;
orange = (num_orange./total).*100;
yellow = (num_yellow./total).*100;
green = (num_green./total).*100;

% Matrix with red, orange, yellow, and green percentages
Percentage_royg = [red; orange; yellow; green]
output = [total, num_red, num_orange, num_yellow, num_green, red, orange, yellow, green];
% h=['total pixels', 'red pixels', 'orange pixels', 'yellow pixels', 'green pixels', 'red %', 'orange %', 'yellow %', 'green %'];
m = [num2cell(output)];
filename = 'PSR Data.csv';
csvwrite(filename, m);
APPENDIX C SUPPLEMENTAL INFORMATION

Reasons why samples were not used in overall analysis:
* Sample ripped in one staple
** Staples were not placed properly causing the load to be placed unevenly
*** Sample believed to be mounted backwards (circumferential in y axis and radial in x axis)
**** Sample completely tore and all tension ratios were not completed

Figure 0-1. Images of biaxial testing of all samples
Figure 0-2. 2nd P-K stress vs Green strain graphs for each individual sample of the 9 treatment groups
Table 0-1. Max areal strain ($\text{AS}_{\text{max}}$), anisotropy index (AI), low (TM$_L$) and high (TM$_H$) tangent modulus for the circumferential and radial direction, and extensibility for the circumferential and radial direction for each individual specimens.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample</th>
<th>$\text{AS}_{\text{max}}$</th>
<th>AI</th>
<th>Circ TM$_L$</th>
<th>Circ TM$_H$</th>
<th>Rad TM$_L$</th>
<th>Rad TM$_H$</th>
<th>Circ Ext</th>
<th>Rad Ext</th>
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<td>Control</td>
<td>FB 1</td>
<td>0.138</td>
<td>0.477</td>
<td>49.9</td>
<td>3375.7</td>
<td>9.4</td>
<td>1048.1</td>
<td>0.216</td>
<td>0.435</td>
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<tr>
<td></td>
<td>FB 2</td>
<td>0.122</td>
<td>0.822</td>
<td>32.4</td>
<td>1252.5</td>
<td>21.5</td>
<td>999.1</td>
<td>0.237</td>
<td>0.293</td>
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<tr>
<td></td>
<td><strong>FB 3</strong></td>
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<td>0.747</td>
<td>19.2</td>
<td>2324.5</td>
<td>23.0</td>
<td>1566.1</td>
<td>0.179</td>
<td>0.239</td>
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<tr>
<td></td>
<td>FB 4</td>
<td>0.087</td>
<td>0.575</td>
<td>38.2</td>
<td>2940.3</td>
<td>16.7</td>
<td>946.1</td>
<td>0.190</td>
<td>0.299</td>
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<tr>
<td></td>
<td><strong>FB 5</strong></td>
<td>0.162</td>
<td>0.766</td>
<td>24.3</td>
<td>3799.7</td>
<td>13.8</td>
<td>1539.5</td>
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<td>0.408</td>
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<td><strong>FB 6</strong></td>
<td>0.088</td>
<td>0.407</td>
<td>74.5</td>
<td>2187.5</td>
<td>21.5</td>
<td>1589.1</td>
<td>0.131</td>
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<td>0.067</td>
<td>0.417</td>
<td>88.1</td>
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<td>44.3</td>
<td>1180.0</td>
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<td>0.573</td>
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Table 0-2. (Cont.)

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<th>$C_{\text{R}}$</th>
<th>$C_{\text{R}}$</th>
<th>$R_{\text{L}}$</th>
<th>$R_{\text{L}}$</th>
<th>$C_{\text{L}}$</th>
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<td>0.208</td>
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<td>0.416</td>
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Table 0-3. Material constants along with the conditional number, $R^2$ of the fit, and anisotropy index (AI) for individual specimens by fitting the experimental data to the Fung-type model.

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<td>FB 7</td>
<td>0.168</td>
<td>53.634</td>
<td>8.830</td>
<td>2.424</td>
<td>0.955</td>
<td>0.201</td>
</tr>
<tr>
<td>Ang-II+ PD123,319</td>
<td>FB 1****</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FB 2**</td>
<td>0.721</td>
<td>14.299</td>
<td>11.003</td>
<td>2.393</td>
<td>0.809</td>
<td>0.803</td>
</tr>
<tr>
<td></td>
<td>FB 3*</td>
<td>1.797</td>
<td>6.176</td>
<td>2.570</td>
<td>2.024</td>
<td>0.820</td>
<td>0.560</td>
</tr>
<tr>
<td></td>
<td>FB 4</td>
<td>0.167</td>
<td>46.462</td>
<td>10.402</td>
<td>-2.232</td>
<td>0.906</td>
<td>0.185</td>
</tr>
<tr>
<td></td>
<td>FB 5</td>
<td>0.296</td>
<td>68.502</td>
<td>9.727</td>
<td>-2.616</td>
<td>0.943</td>
<td>0.108</td>
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<td></td>
<td>FB 6</td>
<td>1.163</td>
<td>17.597</td>
<td>8.951</td>
<td>3.697</td>
<td>0.823</td>
<td>0.594</td>
</tr>
<tr>
<td></td>
<td>FB 7</td>
<td>3.373</td>
<td>14.523</td>
<td>5.380</td>
<td>-0.476</td>
<td>0.750</td>
<td>0.349</td>
</tr>
<tr>
<td></td>
<td>FB 7(8)</td>
<td>1.553</td>
<td>16.063</td>
<td>6.792</td>
<td>1.733</td>
<td>0.747</td>
<td>0.479</td>
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AD.1 Biomechanics

All living organisms experience or produce some type of movement in their system. The study of biomechanics allows scientists and engineers to analyze the biomechanical function of biomaterials, biological systems, organs, tissue, cells, and cell organelles. Understanding the mechanics of the body is important to improve the movements of the body and to understand the alterations in mechanical function the body may suffer after injury or during a disease. By doing so, the research community can develop treatments to improve movement, prosthetics for those that suffered limp loss, medical devices to assist loss of mechanical functions, therapeutic treatments, and biomaterials to replace injured tissue.

AD.1.1 Mechanical properties

The mechanical properties include material and structural properties. Material properties are what make up the material, and structural properties characterize the behavior of the material such as size, shape, or form it undergoes (88). Material properties can be characterized by the relationship between stress and strain. In a linear material, stress and strain are proportional and when graphed are a straight line. A non-linear relationship are when the mathematical relation between stress and strain cannot be characterized by a straight line. A material can be elastic or plastic. When stress is applied and released, and the material reverts to its undeformed state, it is considered an elastic material. In a plastic material, there is a permanent change in strain or deformation when the stress is applied and then released (Figure 0-1) (89). These properties can be tested through mechanical testing methods using various standard laboratory equipment. The stiffness or Young’s Modulus of a linear elastic material can be calculated by measuring the slope of the linear region (Figure 0-1a). The area under the curve is the strain energy, the energy that is dissipated due to the work that is done during deformation (Figure 0-1b) (88).
The key material properties studied are stress and strain. Stress is the force applied to the material per area, and strain is the deformation of the material in respect to a line or square. Stress is calculated by dividing the force by the cross-sectional surface area (A) (Figure 0-2a), and strain is a ratio of the change in length divided by the initial length. The load-displacement curve and the stress-strain curve will have an identical in shape, but the x and y scales will be different (Figure 0-2b,c) (88).
AD.1.2 Stress and strain

Stress can be determined by normalizing the force that is being applied to a cross-sectional area. There are three forms of stress: normal stress, shear stress, and bending stresses. Normal stress is when a material is subject to force under axial loading. Shear stress is when force is being applied in equal and opposite transverse fields. (90). Normal stress is when force is being applied perpendicular to the surface area (Figure 0-3a). There are two kinds of normal stresses, tensile and compressive stress. Tensile stress is when the material is stretched or lengthened (Figure 0-3b), and compression stress is when the material is compressed or shortened (Figure 0-3c). Shear stress is force acting parallel to the surface area (Figure 0-3d).

Strain is the deformation the material undergoes during stress. Normal strain is the deformation the material undergoes by forces acting perpendicular to the surface in respect to a line, normal stress. The material is either elongated by the tensile stress or contracted by the compression stress which act by force being applied to the surface perpendicular to it. Shear strain is the deformation the material experiences by forces acting parallel to the surface with respect to a shear stress. Shear strain changes the angle between the corners of the square which were originally right angles.

(Figure 0-3. Diagrams of (A) normal and (D) shear stress and the two types of normal stress, (B) tensile and (C) compression stress.)
Stress-strain plots allow one to measure various bulk mechanical properties of materials. The strength of the material correlates with the stress required to deform material. The yield strength is the amount of stress that a material can undergo before it transitions to plastic deformation. The largest stress is the ultimate strength that a material can withstand, and the breaking strength, also known as failure strength, is the strength that the material can endure before breaking (Figure 0-4a). The toughness can be measured by calculating the area under a material’s stress-strain curve (Figure 0-4b) (88).

**Figure 0-4.** Strain-strain curve depicting the (A) yield, ultimate, and breaking stress and (B) the toughness of the material, adapted from Saunders (88)

Stiffness can be calculated by acquiring the slope of the linear region of a material’s stress-strain curve, and it allows one to measure the compliance of the material. Materials will deform less if its stiffness is greater. In linear material, the stiffness is called Young’s modulus (E). In a non-linear biological material, there are typically two pseudo-linear regimes, the toe region and the linear region. The stiffness of the material can be assessed via the high and low tangent modulus (TM), stiffness of the linear and toe region respectively (Figure 0-5) (74).
Figure 0-5. Stress-strain curve with Young’s Modulus for a linear material and Tangent Modulus for a non-linear material

In an elastic material, the material will revert to its original state when loaded and unloaded. In viscous materials, the material will not revert to its original state but instead stay at the state in which it was when the load was released. Viscoelastic materials are materials that exhibit both viscous-like and elastic-like properties. The relationship between stress and strain in viscoelastic material depends on time (91).

AD.1.3 Biaxial testing

There are various forms to test material depending on its properties. For incompressible materials such as biological tissues, biaxial testing is ideal as it allows for the characterization of a three-dimensional stress-strain relationship. Biaxial testing is where a material is subjected to load in both the x and y directions in contrast to uniaxial testing which only subject the material to load in one direction.

When running biaxial testing in viscoelastic material, such as biological tissues, hysteresis is present. Hysteresis is the energy lost within the material between successive loading and unloading cycles. It is necessary to precondition the material prior to testing so it reaches an equilibrium state before running biaxial testing (Figure 0-6a), with the loading and
unloading curve taking the same path as it is being displaced. In other words, the material will exhibit more “pseudo” elastic-like properties (Figure 0-6b) (88).

![Diagram](image)

**Figure 0-6.** Preconditioning of material during biaxial testing (A) load vs time and (B) load vs displacement

There are many modes of biaxial deformation. A material can experience tensile stress in both axis (Figure 0-7a), tensile stress in one axis and compression stress in the other (Figure 0-7b), and compression stress in both axis (Figure 0-7c) (92). A material can be stretched to either a particular load or deformation and then allowed to return to 0 load or deformation. The type of biaxial model used is dependent on the load the material experiences in the body.

![Diagram](image)

**Figure 0-7.** Models of biaxial testing, (A) tensile load in both axis, (B) tensile load in one axis and compression in the other, and (C) compression on both axis, adapted from Ozkaya, et. al. (92)
In biaxial testing, strain and stress is measured taking into account deformation. The deformation gradient \( F \) is the fundamental measurement of deformation of a material that mathematically explains the changes in length, angles, and area of volume. **Figure 0-8** demonstrates a simple illustration of the deformations of a square. \( \beta_0 \) is the original position of the object, or the reference configuration, and \( \beta_t \) is the position of the object after deformation, or its current configuration. \( x_1 \) and \( x_2 \) are the axis of \( \beta_0 \), and \( x_1' \) and \( x_2' \) are the axis of \( \beta_t \). A, B, C, and D are the initial \( x_1 \) and \( x_2 \) coordinates, and A’, B’, C’, and D’ are the \( x_1' \) and \( x_2' \) coordinates after deformation. \( u \) is the displacement vector \((x'^{-} - x)\). To calculate deformation, \( F \) needs to express \( x \) in terms of \( x' \) by using Eq. (0.1).

\[
x_1 = ax_1' + bx_2' \\
\]

\[
x_2 = cx_1' + dx_2' \\
\]

\[
F = \frac{dx}{dx'} \\
\]

**Figure 0-8. Simple diagram of material deformation/motion**

Engineering strain can be calculated by dividing the experimental length by the original length. However, in biaxial testing, the Green strain \( (E) \) tensor is calculated using Eq. (0.2).

\[
C = F^T F \\
\]

\[
E = 0.5(C-I) \\
\]

As stated previously, stress is the force being applied to a specific area. However, there are three stresses that consider the orientations, force and area, that give rise by the natural tensorial nature of stress: Cauchy stress, 1\textsuperscript{st} Piola-Kirchoff (P-K) stress, and 2\textsuperscript{nd} P-K stress.
Cauchy stress (σ) is the true stress experienced by the material. The 1st P-K stress (P) is a measurement of stress that is Lagrangian in nature. 1st P-K stress is the most commonly measured stress with mechanical testing equipment. It measures the force being applied at each time point divided by the area of the square in the original configuration, β₀ and is calculated with Eq. (0.3). Cauchy stress is calculated with Eq. (0.4) where J is the Jacobian. The Jacobian is a measure that quantifies the change in volume that is produced during the deformation. If material is incompressible such as tissue, J=1. The 2nd P-K stress (S) is calculated using Eq.(0.5). The 2nd P-K stress is not a direct physical measure of stress, but it is useful in many ways. The 2nd P-K stress is the energy conjugate of Green strain (E) and can thus be used to determine constitutive models (82).

\[ P = \frac{f}{A_0} \quad (0.3) \]
\[ \sigma = J^{-1} P T \quad (0.4) \]
\[ S = F^{-1} P \quad (0.5) \]

The maximum Green strain is used to calculate the areal strain and the strain anisotropy (Figure 0-9a). Areal strain is calculated by multiplying the maximum Green strain in the circumferential and radial directions (\( E_{\text{circ}} \times E_{\text{rad}} \)). The higher the areal strain, the more strain the material can undergo. Strain anisotropy index is calculated by dividing the maximum Green strain in the circumferential and radial direction (\( E_{\text{circ}} \div E_{\text{rad}} \)). A strain anisotropy index close to 1 indicates a more isotropic material, while less or greater than 1 indicates a more anisotropic material. As mentioned previously, the stiffness of a non-linear material can be measured by the low and high tangent moduli, the stiffness of the toe region and the elastic region respectively (Figure 0-9b). The low tangent modulus (TM\textsubscript{L}) is the stiffness in the toe region (pre-transitional) where the fibers are “un-crimping”. The high tangent modulus (TM\textsubscript{H}) is the stiffness in the elastic region (post-transitional) where the fibers have been “uncrimpled” and are now being
stretched (74). Extensibility is calculated by the x-intercept of the high tangent modulus (Figure 0-9c). The higher the extensibility, the more the material can stretch. The TMₗ, TMₕ, and extensibility are measured in both the circumferential and radial directions. The material is stiffer and hence less extensible in the circumferential direction than in the radial direction (74).

Figure 0-9. ²nd P-K stress vs Green strain graph indicating (A) maximum Green strain used to calculate areal strain and strain anisotropy, (B) high and low tangent modulus, and (C) extensibility.

AD.1.4 Constitutive models

As shown previously, strain energy can be calculated by measuring the area under the curve. Strain energy can also be related to provide phenomenological constitutive models for materials. Constitutive models can also provide additional information on the mechanical responses of materials. One important property to note when deciding an appropriate constitutive model is if the material is anisotropic or isotropic. An anisotropic material has different material properties with respect to the direction the load is applied. An isotropic material has the same
material properties regardless of the direction the load is applied. Another property is if the sample is homogenous or non-homogenous. In a homogenous sample, it is made up of the same material throughout its body. In a non-homogenous sample, it is made up of different materials throughout its body (89). In addition, one chooses a model specific to either elastic or viscoelastic materials. In this thesis, we have focused on the development of constitutive models to characterize homogeneous pseudo-elastic materials.

In an isotropic elastic material, there are two common models: Neo-Hookean and Mooney Rivlin. In these models, strain energy \( W \) is a function of strain variants \( I \) which can be calculated using Eq. (0.6).

\[
I_1 = \text{tr} (B) = \lambda_1^2 + \lambda_2^2 + \lambda_3^2 \quad (78)
\]

\[
I_2 = 0.5 \left[ (\text{tr} B)^2 - \text{tr}(B^2) \right] = \lambda_1^2 \lambda_2^2 + \lambda_2^2 \lambda_3^2 + \lambda_3^2 \lambda_1^2 \quad (78)
\]

\[
I_3 = \text{det} (B) = \lambda_1^2 \lambda_2^2 \lambda_3^2
\]

\[
I_4 = C_{ij} (n_c)i(n_c)j
\]

\( \lambda_1, \lambda_2, \text{ and } \lambda_3 \) are the principal stretches and the left Cauchy-Green tensor \( B \) can be calculated by \( B = FF^T \). \( n_c \) refers to the principle direction of the material fibers. The standard constitutive model for an incompressible, isotropic, elastic material can be determined with Eq (0.7).

\[
\sigma = -p 1 + 2 \frac{dW}{dI_1} B - 2 \frac{dW}{dI_2} B^{-1} \quad (0.7)
\]

\( p \) is a hydrostatic pressure term associated with the incompressibility constraints and \( \sigma \) is Cauchy stress. The Neo-Hookean strain energy can be obtained with Eq. (0.8), and the Mooney-Rivlin (M-R) strain energy can be obtained with Eq. (0.9).

\[
W = 0.5 \mu (I_1 - 3) \quad (0.8)
\]
\[ W = 0.5\mu [ (I_1 - 3) + (1 - \alpha)(I_2 - 3)] \]  \hspace{1cm} (0.9)

\( \mu \) is the shear modulus constant for infinitesimal deformations, and \( \alpha \) is a dimensionless constant. \( \mu \) must be greater than 0, and \( \alpha \) must be greater than 0 or less than 1 (78).

In addition to the M-R strain energy seen in Eq. (0.9), there are various modified forms. Two modified Mooney-Rivlin strain energy equations are the isotropic and anisotropic M-R models. The isotropic modified M-R strain energy can be given by Eq. (0.10). The anisotropic M-R model has an additional anisotropic term and can be given by Eq. (0.11) (80).

\[
W = c_1 (I_1 - 3) + c_2 (I_2 - 3) + D_1 [\exp(D_2(I_1 - 3)) - 1] \quad (0.10)
\]

\[
W = c_1 (I_1 - 3) + D_1 [\exp(D_2(I_1 - 3)) - 1] + (k_1/2k_2) [\exp(k_2(I_4 - 1)^2) - 1] \quad (0.11)
\]

There are various models used for viscoelastic anisotropic material including the Fung-type model and the Choi-Vito model. The strain energy for the Fung-type model can be obtain with Eq. (0.12), and the strain energy from the Choi-Vito model is given by Eq. (0.13).

\[
W = 0.5c (e^{\Omega_1}) \quad (0.12)
\]

where \( Q = c_1 E_{11}^2 + c_2 E_{22}^2 + 2c_3 E_{11} E_{22} \).

\[
W = 0.5c (e^{\Omega_1} - e^{\Omega_2} - e^{\Omega_3} - 3) \quad (0.13)
\]

where \( Q_1 = c_1 E_{11}^2, Q_2 = c_2 E_{22}^2, Q_3 = c_3 E_{11} E_{22} \). c*c1 and c*c2 are metrics for nonlinear stiffness in the circumferential and radial direction respectively, and c3 is the interaction between the circumferential and radial direction (82).