University of Sheffield

# Design and Development of a Robotic Tool for Tissue Engineering Purposes



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### Abstract

This thesis presents a novel robotic bioreactor designed to cultivate cell-seeded constructs. The bioreactor is capable of stiffness-based control, stimulating tissue growth and development through uni-axial mechanical loading. The bioreactor system consists of an actuator, a force sensor, a tissue culture chamber, and a control system capable of monitoring and adjusting stimulation of a cell-seeded construct depending on the real-time stiffness of the growing tissue. The system was designed to provide adaptive stimulation which could improve the growth and development of engineered tissues, with the aim of improving the quality and functionality of the resulting tissue constructs. The system was evaluated using a range of bench-top experiments to characterise the displacement accuracy, force measurement accuracy, and the chamber's ability to isolate the tissue construct from the external environment. Further experimentation into implementing stiffness-based control and in vitro protocols was also explored. The thesis concludes with a discussion of the system's advantages and disadvantages, with emphasis on the future steps that need to be taken to achieve a working robotic bioreactor. Overall, the robotic bioreactor represents a transformative advancement in the field of tissue engineering, providing a new tool for the controlled stimulation of cell-seeded constructs.

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## Chapter 1

# Introduction

Humans strive to improve the human condition, which is why medicine has progressed so rapidly throughout history. Transplants have been a popular solution to tissue damage or degeneration. There are different types of transplants defined by their origin in relation to the recipient patient; the tissue can be transplanted from one site of the



**Figure 1.1:** General tissue engineering protocol is based on cell isolation from an organism, which is then seeded onto a construct. The construct is then stimulated and eventually transplanted into an organism. Based on the figure in [1].

patient to another (autograft), transplanted from one patient to another (allograft) or from another species to a human (xenograft). There are many drawbacks of transplantation, such as the limited number of donor tissue and organs, tissue rejection, and complications associated with immunosuppressant drugs. Biomaterials have been used as substitutes for tissue, but cannot replicate the complex microstructure of tissue in its entirety and therefore cannot match tissue functionality.

Tissue engineering is a relatively recent research area that exploded with its promising potential to replace human tissue. It is considered to be part of regenerative medicine and normally involves combining scaffolds, cells, and biological molecules to form tissue close to native tissue. The term was first introduced in 1988 [21] but has been since repeatedly redefined with the progression of the field. The analysis of specific cell types enables a thorough examination of cells or tissues, which would otherwise be impractical in a complete organism. This approach offers the added ethical benefit of avoiding animal testing. By focusing on isolated cells or tissues, it becomes possible to gain a deeper understanding of their behavior and how it influences the overall organism. This methodology, referred to as in vitro testing, provides valuable insights into the intricate workings of cells and tissues, and can also assist in establishing optimal testing conditions for subsequent animal studies, including those involving implantable devices.

Extensive in vitro experimentation has been conducted into the cellular response to environmental cues, such as mechanical and chemical. Cell types such as cartilage, nerve [22], cardiac tissue [23] and skeletal muscle [24] have all been explored and the effect of mechanical stimulation presented. Mechanical cues are vital for cell survival, especially for cells found in load-bearing areas of the body, such as joints. Mechanical stimulation not only promotes cell proliferation, but it also can affect the body's fibrotic response [24, 25]. The mechanical stimulation of cells is typically achieved with the use of bioreactors. Bioreactors are frequently used as a tool in tissue engineering to mimic the native environment of the cells and improve the mechanical properties of tissue.

Although tissue engineering holds immense promise for building tissues and organs from the ground up, there are several acknowledged challenges within the field. The key lies in identifying and implementing an optimal protocol to stimulate a group of cells, fostering their development in a manner that accurately replicates the geometrical and mechanical characteristics of the desired native tissue [26]. Advancements in tissue engineering fundamentals push regenerative medicine closer to clinical applications and therefore help improve patient treatment and outcome.

This project focuses on the development of a robotic tissue engineering tool to sense and stimulate cell development. This could progress scientific understanding of how mechanical stimulation impacts tissue growth. Adaptive stimulation in tissue engineering refers to the application of stimulation to cells or tissues in a manner that responds to their physiological behaviour. The purpose of adaptive stimulation is to enhance tissue growth, differentiation, and regeneration by providing stimuli that mimic the in vivo environment. The use of robotics in tissue engineering is fairly limited, as it requires an interdisciplinary interest in both robotics and tissue engineering. Tissue engineering traditionally relies on regimented protocols to achieve results. Variables within these protocols require long and time-consuming iterative experiments in search of globally optimum conditions, as current tissue engineering tools are limited by a lack of sensing and adaptive control methods. However, the integration of robotics into this field could automate response to the changes in a living construct, such as a cell-seeded scaffold.

This project raises the research questions: Can a tool that can respond to the changing global mechanical properties of tissue-engineered construct be beneficial to the tissue engineering community?

### **1.1** Research Aims and Objectives

The key aim of the project is to develop an advanced robotic tool for tissue engineering, where tissue properties can be monitored and controlled dynamically.

The project's overarching aim will be accomplished through the following list of objectives:

- (A) Design and manufacture a Bioreactor which can support cells in vitro, stimulate a cell-seeded scaffold and has force sensing capabilities.
- (B) Conduct benchtop validation of the robotic bioreactor to ensure the functionality of the device. Validation focuses on; displacement error, force error, and chamber function.
- (C) Incorporate real-time monitoring into a robotic bioreactor to analyse the global development of material properties of a cell-seeded scaffold throughout an experiment.
- (D) Develop a stiffness-based control system in the robotic bioreactor.
- (E) Test and compare sterilisation protocols to ensure sterilisation is achieved and maintained in the bioreactor chamber ready for in vitro experimentation.

In summary, the research question I would like to investigate is: Can a robot be used as a tool to understand and optimise the time-related correlation between tissue stimulation and tissue development? The inspiration behind this study arises from the hypothesis that when a cell-seeded scaffold changes its mechanical properties throughout an experiment, the force necessary to provide consistent strain also varies. There are a few reasons why the mechanical properties would change over time. Firstly, the initial mechanical properties of the cell-seeded scaffold sample are entirely dependent on the substrate on which the cells are growing, in our case, the scaffold. Over time, as the cells divide during culture they make more cellular material. As these cells mature, they also generate new extracellular matrix which creates a structure between the cells. This increase in material contributes to the alteration of the mechanical properties of the sample. Secondly, the substrate that the cells grow on may be biodegradable and as a result, will break down over time. As stated earlier, initially the mechanical properties depend on this substrate and therefore depend on the degradation rate of the scaffold, which can range from taking weeks to years to fully degrade. Therefore, as the substrate degrades the mechanical properties will also change.

The primary contribution of the project is the development of the robotic bioreactor design and function in two key prototypes, the first and second generation. A further contribution is a proposal and implementation of stiffness-based control.

### **1.2** Thesis Outline

Fig. 1.2 summarises the thesis outline and which chapters work towards the project aims outlined in Sec. 1.1.



Figure 1.2: Thesis summary.

- Ch. 2 outlines the state of art in relevant fields and helps identify the clinical and technical requirements of the project. Sec. 2.1 discusses general tissue engineering fundamentals. As the project is focused on developing a bioreactor equipped with mechanical stimulation, how cells respond to mechanical stimulation and soft tissue mechanical properties is then discussed. Sec. 2.2 and Sec. 2.3 discuss how Scientists can change different parameters in tissue engineering experiments to modify experiment outcomes and tools used to achieve such experiments. Sec. 2.4 then gives a summary of medical and soft robotics and then a deeper view into the uses of robotics for tissue engineering purposes.
- Ch. 3 is the first technical chapter of the thesis and the design criteria for the robotic bioreactor mechanical and electrical design is introduced. Sec. 3.2 and Sec. 3.3 outline the development of the robotic bioreactor mechanical and electrical design with two prototypes. Development of the second prototype was informed by experimentation of the first, therefore it is expected that the second design outperforms the first. This chapter partly addresses objective A described in Sec. 1.1.
- Ch. 4 introduces the development of the robotic bioreactor control. The control design criteria are stated and then Sec. 4.2 introduces two control methods implemented in the robotic bioreactor: force and position control. The bioreactor's capabilities to execute various stimulation regimes are exhibited which shows how versatile the tool is. This chapter also addresses objective A described in Sec. 1.1.
- Ch. 5 aims to characterise and directly compare the performance of the robotic bioreactor prototype to determine the design to continue with the net stages of the robotic bioreactor development. Benchtop experiments to test the displacement accuracy, force measurement and chamber function. This chapter addresses objective B described in Sec. 1.1.
- Ch. 6 starts the development towards stiffness-based control. Sec. 6.1 describes the design and use of a controllable stiffness scaffold phantom to determine how to accurately measure the stiffness of a tissue construct during an in vitro without interrupting the experiment. A soft phantom scaffold is introduced in Sec. 6.2 and the stiffness is tracked using the stiffness calculation method determined in Sec. 6.1. This chapter addresses objectives C and D described in Sec. 1.1.
- Ch. 7 presents the development of the robotic bioreactor in vitro protocols that works towards addressing objective E described in Sec. 1.1.
- Ch. 8 concludes the thesis and discusses a number of potential directions for future work.

These chapters will aim to address the research aims and objectives stated above. However, developing a tool for tissue engineering is a complex process that involves multiple challenges which must be considered. Natural tissue is highly complex and dynamic, with multiple factors, including mechanical, electrical, and chemical cues, affecting cell behaviour. Therefore, creating a tool that can replicate these conditions accurately can be challenging. The way to address this is to use the literature to inform conditions that commercial bioreactors can achieve as a minimum and then build on these functions. This also enables the comparison of the robotic bioreactor function to commercially available bioreactors. Tissue engineering tools need to be scalable and reproducible, meaning they should be able to produce consistent results across different batches and quantities. This is especially important when developing tissues for clinical use. Tissue engineering tools need to be biocompatible, meaning they should not cause any adverse reactions or harm to cells or tissues. Using materials that are known to be biocompatible and also doing in-house toxicity testing will ensure the components of the devices will not cause any adverse effects to the cells. Ensuring biocompatibility requires the use of materials and manufacturing processes that are safe and do not interfere with the growth and development of cells. Different tissues have unique characteristics and requirements, and tissue engineering tools need to provide specific cues that promote the growth and development of different cell types. Optimizing these cues can be challenging, as it requires an understanding of the complex interactions between cells and their microenvironment. To start with, using cues defined and explored in literature can give a base to build on with the robotic bioreactor.

Overall, the impact of adaptive stimulation in tissue engineering is promising, as it offers a new approach to developing functional and mature tissues that can be used for a range of medical applications, including tissue repair, organ replacement, and drug screening. A robotic tool that can provide this adaptive stimulation can help researchers conduct extensive and repeatable experiments, with less time-consuming iterations.

### Chapter 2

## Literature Review

This section aims to give a broad overview of the fields associated with this project. First, tissue engineering fundamentals are discussed and then we move on to common and state-of-the-art techniques and tools used in tissue engineering. Next, the field of robotics is broadly discussed and then the scope is narrowed to robotics in tissue engineering and where the current gaps in this field are. Finally, where the future of robotics in tissue engineering is discussed and speculated about.

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The benefits of tissue engineering are numerous and include;

- 1. Regenerative potential: Tissue engineering offers a promising approach to regenerating damaged tissues or organs, potentially eliminating the need for organ transplantation or reliance on lifelong medication [27].
- 2. Improved drug development: Tissue engineering can provide more accurate and reliable models for drug testing, reducing the need for animal testing and potentially accelerating the drug development process [28].
- 3. Reduced risk of rejection: Tissue engineering can produce tissue or organs that are less likely to be rejected by the patient's immune system since they can be designed using the patient's own cells [29].
- 4. Ethical benefits: Extensive tissue engineering preliminary studies can help reduce the number of laboratory animals used in studies [30].

Tissue engineering can improve the quality of life for patients suffering from a wide range of medical conditions, from chronic wounds [31] to organ failure [32]. In addition, tissue engineering has the potential to bring significant societal impacts that go beyond the medical and healthcare fields. Firstly, there are ethical issues that need to be openly discussed to ensure that tissue engineering practices adhere to ethical standards [33]. As tissue engineering often involves the use of human cells or tissues, ethical considerations arise around the use of embryonic stem cells, human cloning, and the creation of chimeras or hybrid organisms [34]. Secondly, with the growing demand for tissue-engineered solutions in medicine, technologies may give rise to new industries and job opportunities, such as bio-printing and cell manufacturing. Tissue engineering could help improve public health by providing solutions for a wide range of diseases and conditions that currently lack effective treatments. This, in turn, could lead to reduced healthcare costs, increased productivity, and improved quality of life.

Although significant progress has been made in tissue engineering, there are several challenges that still need to be addressed. Firstly, choosing the right biomaterials for tissue engineering is critical. The ideal biomaterial should be biocompatible, biodegradable, and have mechanical properties similar to the native tissue [35]. Tissue engineering strategies should aim to replicate the specific function of the target tissue. This requires a detailed understanding of the structure and function of the native tissue. In addition, early engineered tissues did not match the material properties of native tissue exactly [36], although this has improved significantly [37]. This is problematic as when such materials are implanted into the patient, the engineered tissue will not perform as well as native tissue and has a high risk of failure or damage [38]. This will cause pain and discomfort for the patient and is likely to lead to revision surgery which would disrupt the patient's life. The lack of blood vessels in engineered tissues is a major challenge [39]. Tissue engineering strategies should aim to promote angiogenesis and vascularization to ensure adequate nutrient and oxygen supply to the tissue [40, 41]. Engineered tissues are often recognized as foreign by the immune system, which can lead to rejection. Strategies to minimize the immune response are critical for the success of tissue engineering [42]. Current tissue engineering methods are often labour-intensive and time-consuming, making it difficult to produce largescale, clinically relevant tissues. Strategies for scale-up and automation are needed to enable widespread clinical application [43]. Tissue engineering products must undergo rigorous regulatory approval before they can be used in clinical settings and meeting these regulatory requirements is a significant challenge for tissue engineers [44].

### 2.1 Tissue Engineering Fundamentals

Tissue engineering is a multidisciplinary field that applies principles from biology, engineering, and medicine to create functional living tissues that can be used to repair or replace damaged tissues in the body [45].

There are two key paths to consider when discussing tissue engineering, and they will described using ex vivo and in vivo. In vivo tissue engineering involves the regeneration of tissues within the body of the patient, while ex vivo tissue engineering involves the manipulation of cells or tissues outside of the body, often with the goal of implanting the engineered tissue back into the patient.

In vivo tissue engineering is a promising approach for the regeneration of damaged or diseased tissues. This approach involves the use of biomaterials, growth factors, and cells to stimulate tissue regeneration within the body of the patient. In vivo tissue engineering can be used to regenerate a variety of tissues, including bone, cartilage, skin, and blood vessels. One of the advantages of in vivo tissue engineering is that it can be performed without the need for invasive surgery, which can reduce the risk of complications and improve patient outcomes.

Ex vivo tissue engineering, on the other hand, involves the manipulation of cells or tissues outside of the body, often with the goal of implanting the engineered tissue back into the patient. This approach can involve the use of scaffolds to support cell growth and tissue regeneration, as well as the use of growth factors and other biomolecules to stimulate tissue regeneration. Ex vivo tissue engineering can be used to regenerate a variety of tissues, including bone, cartilage, skin, and blood vessels. One of the advantages of ex vivo tissue engineering is that it allows for the precise control of the microenvironment in which the cells are grown, which can improve the quality and consistency of the engineered tissue.

Tissue engineering begins with the selection of an appropriate cell source. Cells can be harvested from a patient or donor, or they can be derived from stem cells or other sources. The selected cells should be capable of proliferating, differentiating, and functioning in a tissue-specific manner [45]. The engineered tissue must be evaluated to ensure that it is functional and safe for use in humans. Evaluation can involve a variety of techniques, such as mechanical testing, histological analysis, and functional assays, to assess the properties and performance of the engineered tissue. Scaffolds are three-dimensional structures that provide a platform for cells to grow and organize into functional tissues [46]. Scaffolds can be made from a variety of materials, such as synthetic polymers, natural polymers, or decellularised tissues. The scaffold should have the appropriate physical and chemical properties to support cell attachment, proliferation, and differentiation. Bioreactors are specialized devices that provide the necessary environmental cues, such as mechanical stress, oxygen and nutrient supply, and biochemical signals, to promote tissue development and maturation. Bioreactors can be designed to replicate the physiological conditions of the tissue being engineered, and they can be used to optimize tissue formation and function [47].

Overall, the fundamentals of tissue engineering involve the selection of an appropriate cell source, the design and fabrication of a scaffold that supports cell growth and tissue formation, the provision of appropriate environmental cues and biochemical signals and the evaluation of the engineered tissue to ensure its safety and efficacy.

#### 2.1.1 Cells and their environment

Cells are the fundamental building blocks from which all living things are made of. They contain various specialised structures to carry out important roles that differ depending on cell function and location. The appropriate cellular micro-environment is crucial and influences the behaviour of the cells [48]. It becomes even more critical when working in vitro, as cells do not naturally occur in isolation. Working with isolated cells is, however, inherent to in vitro investigation and therefore, the closer the rest of the micro-environment is to native conditions, the closer the action of the cells is to the native cell action. The structure of an animal cell is shown in Fig. 2.1. The main structures inside a cell are; the nucleus, the cell membrane and the cell cytoplasm. The nucleus contains the genome of a cell and regulates the activities of the cell. It is often referred to as the brain of the cell as it is integral to protein and enzyme synthesis, cell division, cell growth, storage of genetic information and hereditary characteristics. The cell membrane isolates the cell from its external environment. The semipermeable lipid bilayer controls the transport of materials, such as ions, nutrients, wastes and metabolic products, across the membrane and it is also able to keep toxins outside of the cell.



Figure: Animal Cell Structure with Cell (Plasma) Membrane, Image Copyright Sagar Aryal, www.microbenotes.com

Figure 2.1: Structure of an animal cell. Figure from [2].

The cell membrane anchors the cytoskeleton to hold the cell together and also anchors the ECM and other cells to form tissues. To allow the transport of molecules across the membrane, processes such as passive osmosis/diffusion, transmembrane protein channels, endocytosis, and exocytosis are used.

Cell organelles, such as ribosomes, endoplasmic reticulum, Golgi apparatus, chloroplasts and mitochondria, are found amongst the cytoplasm and are separated from the cytoplasm by enclosed membranes. The cytoskeleton provides the cytoplasm with structure and consists of filamentous protein structures; microtubules, intermediate filaments and actin filaments. The cytoplasm has three main functions; 1) forms connections from the external environment to the cells, 2) organises the contents of the cell [49] and 3) generates forces to move and change the shape of the cell through polymerisation and depolymerisation of the actin filaments and microtubules along with molecular motor action [50].

The extracellular matrix (ECM) fills the spaces between cells and constitutes a huge part of the cellular microenvironment and is shown in Fig. 2.2. It is a non-cellular complex network that surrounds and provides structural support to the cells and is said to determine the stiffness of the tissue [51]. Stiffness is a property that describes the resistance of a material or structure to deformation under an applied load [52]. The main components of the ECM, including collagen and elastin, are produced and secreted by the surrounding cells. The relationship between the cells and their environment is thought to be bidirectional; cells produce and rearrange ECM components and the ECM regulates and modifies cell action [53, 54]. Cells in living tissues communicate with each other through a complex network of biochemical signals, such as growth factors, cytokines, and extracellular matrix (ECM) proteins. Mechano-biochemical interactions in the extracellular matrix (ECM) play a crucial role in wound healing and disease progression. During wound healing, cellular forces and physical forces applied to the ECM mediate matrix patterning and remodelling. The ECM provides mechanical support and biochemical signals that regulate cell migration, proliferation, and differentiation. The ECM also plays a role in angiogenesis and tissue repair, and its interactions with cells and growth factors are critical for functional tissue repair. Dysregulation of ECM remodelling can lead to pathological conditions such as fibrosis [55, 56]. Understanding the complex interplay between cells, ECM, and mediating molecules is essential for developing effective treatments for wound healing and disease progression. In tissue engineering, researchers use a variety of approaches to provide the appropriate biochemical signals to promote cell growth, differentiation, and tissue formation.



Figure 2.2: The extracellular matrix consists of a network of proteins and carbohydrates. Figure and caption from [3].

The conditions in which the cells are kept must also be optimum. A medium must

be added to provide nutrition to the living cells to support their survival. Dulbecco's modified eagle's medium (DMEM) with Fetal Calf Serum, penicillin, fungizone and glutamine is a common example of such a medium [57]. Cells also must be kept in strict conditions (temperature, humidity, carbon dioxide and oxygen content of the atmosphere) to support their survival.

#### 2.1.2 Mechanobiology

Hooke was responsible for the discovery of the law of elasticity, better known as Hooke's Law (F = kx), which describes how a force, F, needed to extend a spring, k, a distance, x, is linearly proportional to that distance [58]. He also was the first to observe a cell under a microscope. Two fields that were very different are now known to be intertwined in the field of Mechanobiology.

All cells are deemed to be mechano-sensitive, meaning that cells respond in some way to mechanical stimuli [59]. Mechanobiology focuses on research in the physical forces and changing mechanical properties of cells and how these factors induce cell response. These cell responses can be tuned by altering the magnitude and frequency of forces applied to the tissue. Mechanobiology opens doors into "therapeutic interventions that reduce and reverse injury to damaged tissues" [60]. The loading that cells are naturally exposed to in the body environment is deemed vital for their development and survival [61].

Mechanotransduction is the process by which "physical cues are translated into biochemical signals" and is crucial to normal cell activity [62]. There are two types of forces to consider in mechanotransduction, external and internal to the cell. We can use the concept of mechanotransduction to explain how we hear sound and feel pressure, where vibrations or force are converted into electrical signals which can be translated by the brain. Cells also use forces, such as shear, compression and tension, to mediate cell activity. There have been various techniques developed to estimate the forces that cells exert using the fundamental of Hooke's law, such as traction force microscopy [63], micro post arrays [64] and atomic force microscopy [65]. The extent to which Hooke's law is followed depends on the cells and tissue in question as Hooke's law describes homogeneous, isotropic, linear elastic materials and biological tissue are characteristically heterogeneous, anisotropic, time-dependent and non-linear which makes classic material model assumptions difficult. Fig. 2.3 shows how biological structures can differ from engineering materials in stress-strain relationships. There have been some viscoelastic models developed that try to represent tissue [66, 67, 68, 69], but they have their limitations.

To detect tension within the cell, molecular sensors such as Fluorescence-Resonance Energy Transfer (FRET) based force sensors have been developed that measure tension using intracellular probes [70].

$$stress(\sigma) = \frac{F}{A}$$
 (2.1)

where F is force (N) and A is area  $(m^2)$ .

$$strain(\epsilon) = \frac{L - L_0}{L_0} \tag{2.2}$$

where L is the length after a force is applied and  $L_0$  is the original length.





**Figure 2.3:** Comparison of the stress-strain relations is different for typical engineering materials and biological materials. Figure based on [4].

#### 2.1.3 Soft Tissue Mechanical Properties

Soft tissue is very complex and is constructed of many different layers, all of which have their own mechanical properties. This is one reason as to why it becomes quite difficult to simulate their behaviour. In this context, soft tissue refers to tissue in the body that is not hardened by processes of ossification or calcification such as bones [71].

Soft tissue show viscoelastic, incompressible and usually anisotropic. A viscoelastic material is one that has both the properties of an elastic and a viscous material and

has been used to model the material properties of soft tissues. The Maxwell, Voigt and Zener models are linear viscoelastic models and are shown in Fig. 2.4. The Maxwell model consists of a series connection between a linear Hookean spring and a Newtonian dashpot (Eq.2.3). The Kelvin-Voigt model can also be used, which models a linear Hookean spring and a Newtonian dashpot in parallel to each other (Eq.2.4). The Zener model is modelled from a linear Hookean spring in parallel with a Newtonian dashpot and a Hooksean spring in series (Eq.2.5). The forces needed to displace a viscoelastic material are time-dependent which means that there is a delayed response to a stimulus and a loss of energy inside the material.



**Figure 2.4:** Mechanical equivalents of the Maxwell, Voigt and Zener models that use Newtonian dashpots and linear Hookean springs.

$$F + \frac{\beta_1}{k_1}\dot{F} = \beta_1 \dot{x} \tag{2.3}$$

$$F = k_2 x + \beta_2 \dot{x} \tag{2.4}$$

$$F + \frac{\beta_3}{k_4}\dot{F} = k_3x + \frac{\beta_3(k_3 + k_4)}{k_4}\dot{x}$$
(2.5)

Although these linear viscoelastic models demonstrate viscoelastic behaviour well, they do not demonstrate soft tissue's highly variable and dynamic structures. Fung's quasi-linear viscoelastic (QLV) model [72] has been widely used for the modelling of viscoelastic properties of soft tissue. The QLV model incorporates both the time dependency and the nonlinearity of soft tissue in a simplified integral model. The model does have limitations, one of the main being that it does not always provide physically reasonable behaviour and is appropriate for "appropriate materials whose relaxation-rate coefficients are weakly dependent on deformation, or where the deformation comprises small perturbations about a large initial deformation" [73].



**Figure 2.5:** Cellular structures involved in mechanotransduction. Based on the figure in [5].

Many mechanotransduction mediators have been identified throughout the years with the advancement of biological technologies and an overview of these structures and pathways is shown in Fig. 2.5. Cadherins are the main proteins at intercellular junctions and bind cells together like a 'biochemical velcro' [74]. They can sense the tension between cells and therefore play a key role in mechanotransduction. The cell membrane is the primary site of force transmission. Focal adhesions are dynamic protein structures that form mechanical links between the cytoskeleton and the ECM. They are responsible for cell shape stability, environmental sensing and signalling and force transduction. Both integrins and cadherins respond to external forces direct cell behaviour and tissue homeostasis. How these proteins respond to force is still up for debate but some theories revolve around forces changing the protein conformations and exposing hidden binding sites [75]. Integrins act as mechanoreceptors and transmit mechanical signals to the cytoskeleton, and they physically connect the cytoskeleton to the extracellular matrix, thus transmitting physical forces. Integrins are involved in the mechanotransduction process in cells of the musculoskeletal system and mechanoresponsive cells in other tissues [76]. Integrins also regulate focal adhesion formation and clustering, and they are associated with signalling proteins such as focal adhesion

kinase [77]. Internal forces can also be produced by the cell. When a cell extends its membrane or rearranges its actin cytoskeleton, internal endogenous contractile forces are produced and can stimulate cell action [78].

When selecting the type of cells to use for tissue engineering experiments using mechanical stimulation, a number of considerations are taken into account: ease of use, growth rate, accessibility and relevance to the research. Using cells derived from human tissue requires ethical approval in line with the Human Tissue Act 2004 [79] and so choices are then limited to whether ethical approval is granted. However, immortal human cell lines have been created and can be used in research which does not now require the user to obtain ethical approval, these are often derived from cancers and have been in use for many years [80]. Fibroblasts are commonly used in tissue engineering research as cell sources, as they are more easily isolated than other cells, easily differentiated, able to be preserved over a long period of time and have existing protocols for isolation [81]. They can also be obtained from any connective tissue so have many potential sources, examples including cardiac fibroblasts, bronchial fibroblasts, rotator cuff fibroblasts and dermal fibroblasts. Waste tissue from surgery is a common source of this cell. Fibroblasts are active forms of fibrocytes, which are commonly found in the connective tissue and are key in the wound healing process [82]. They are responsible for collagen and ECM synthesis and provide structural integrity for tissue by continuously secreting precursors of the ECM. Understanding how the cells behave under mechanical strain is important, as they are going to be the main cell in vivo for the synthesis and repairing of tissue, also they have an important role in making biohybrid scaffolds. Research into how the source of fibroblasts can affect their activity shows they are comparable across different sources [83].

Mechanical stimulation is a technique used to apply physical forces to cells in order to mimic the in vivo environment and promote tissue growth and development. Bioreactors are commonly used to apply mechanical stimulation to cells in vitro. Bioreactors can provide a dynamic environment for cell culture by applying perfusion, tensile or compressive loading, rotation, or other conditioning. Different types of bioreactors have been developed for mechanical stimulation of cell and tissue cultures, including custombuilt bioreactors [84], self-designed bioreactors [85] and computer-regulated bioreactors [86]. Mechanical stimulation has been shown to enhance the mechanical, structural, and cellular properties of tissue-engineered constructs [87]. In addition, mechanical stimulation has also been shown to rescue degenerated tissues. In [88], the authors found that cyclic mechanical stimulation (6% strain, 0.25 Hz, 8 h/day) in a bioreactor system rescued the Achilles tendon from degeneration and restored pathological changes and mechanical properties to levels seen in healthy tendons. Bioreactors that provide physiologically relevant loading, scaffolds, and growth factors are important for creating an appropriate mechanical context for tissue-engineered constructs [89]. A study by [90] showed how mechanical stimulation alone can also initiate chondrogenesis. Overall, bioreactors are an important tool for applying mechanical stimulation to cells in vitro and promoting tissue growth and development.

#### 2.1.4 Types of Experimentation

There are different types of experiments used in tissue engineering: in vivo, in vitro, ex vivo and in silico. In vivo is Latin for "within the living" and describes the experimentation with or within whole organisms. In vitro is Latin for "in glass" and describes the experiments conducted outside of a living organism, for example with specific cells in laboratories. The utilisation of in vitro testing provides a platform for conducting a comprehensive analysis of cells or tissues, surpassing the limitations imposed by studying the entire organism. Additionally, it offers the ethical advantage of avoiding the need for animal testing. This approach enables a deeper understanding of the isolated behaviour of tissues or cells, and therefore, enables a clearer understanding of how such behaviour contributes to the overall organism. Ex vivo is Latin for "out of the living" and is used to describe experiments in or on tissue from an organism that has been extracted from the organism in an external environment. In silico is used to describe tests that are performed on a computer or via computer simulation. The primary advantages and disadvantages of each type of experiment are outlined and discussed in Tab. 2.1.

Type	Benefits	Drawbacks
In Vivo	Investigate the effects of an exper-	Ethical Implications of animal test-
	iment on a whole organism. Com-	ing. Cannot isolate specific cell
	plex multicellular response.	activity. Harder to control spe-
		cific variables Differences between
		responses depending on the organ-
		ism tested - so not always represen-
		tative of human response
In Vitro	Ability to isolate the behaviour of	Ethical Implications of animal test-
	one cell type to study. Ethical ad-	ing. Cannot isolate specific cell
	Vantages of avoiding animal testing.	activity. Harder to control spe-
	automated Controlled and repeat	responses depending on the organ
	altoinated. Controlled and repeat-	ism tested - so not always represen-
	able methodology.	tative of human response & Can-
		not mimic the native cell environ-
		ment precisely. Challenging to ex-
		trapolate in vitro results back to
		the whole organism
Ex Vivo	Allows more controlled conditions	Short observation time. Less re-
	to be set for experimentation on	active biological response to treat-
	cells or tissues than in vivo. Able	ment stimuli. Tissue from animals
	to perform tests or measurements	can still respond differently to that
	considered to be unethical or not	of humans
	possible in living organisms. Main-	
	tains complexity of tissue structure.	
In Silico	Ethical advantages of avoiding ani-	Not using real cells/organisms and
	mal testing.Can be used as a tool	so real cell responses and be-
	to identify the best protocol to	haviours cannot be truly repre-
	follow. Allows controlled testing	sented. Some behaviours and cell
	ot many variables.Can model rela-	interactions are not fully under-
	tively complex interactions e.g. be-	stood and so cannot be tested in
	tween multiple cell types	silico

**Table 2.1:** Table to describe the benefits and drawbacks of In Vivo, In Vitro and Ex Vivo and In Silico experimentation methods

### 2.1.5 Scaffolds

Most tissue-derived cells are anchorage-dependent and need surface support for normal proliferation. Scaffolds are used to mimic the ECM of the native tissue and their
purpose is to form a structure in which the cells can attach, proliferate, and migrate along. Scaffolds can be manufactured from natural or synthetic materials.

Natural scaffolds are those which are formed from materials derived from nature, such as Alginate, a material derived from seaweed, which can be used as a scaffold and has been shown to support cell growth via cell encapsulation [91]. Inorganic ceramics are also regarded as natural scaffolds, examples include calcium phosphates and inorganic polymers (e.g. polysaccharides, lipids and proteins) [92]. Natural scaffolds are advantageous as their structure is similar to native tissue. There are, however, various drawbacks of using natural materials to form scaffolds which should be considered; i) they need donor tissue, so availability is limited to the number of donors available, ii) there is a risk of pathogen transmission from the donor and recipient and iii) a high degree of variation between batches [93]. In addition, decellularisation of the scaffold, which is needed to prevent an immune response, can damage the structure beyond use. Synthetic scaffolds are those which are fabricated in the laboratory. There are various types and the material is selected depending on the cell type and function. Biodegradable polymers are ideal candidates to synthesise a scaffold, as they have the ability to deteriorate through hydrolysis and do not produce any toxic by-products[94]. There are some common synthetic polymeric materials commonly used for scaffold; such as polylactic acid (PLA), polyglycolic acid (PGA)[95], and poly(glycerol sebacate) (PGS) [96]. They are beneficial as they can be fabricated relatively cheaply with tailored physical properties and degradation rates [97]. For example, the degree of methacrylation (DM) was found to directly affect the rate of degradation of Poly(glycerol sebacate)-Methacrylate (PGS-M) [98] and by changing this degree of methacrylation, this changes the degradation rate. PGS-M is a photocurable form of Poly (glycerol sebacate) (PGS) [98] which has been reported to support cell survival for at least 7 days [98].

One last consideration of scaffolds is that they need to be sterilised before they can be seeded with cells to prevent contamination during experiments [99]. There are a few gold-standard sterilisation methods, including autoclaving and plasma treatments. Autoclaves commonly used in numerous industrial processes, use steam sterilisation to remove all forms of life and other biological agents. The standard steam sterilisation protocol is 15psi, 121°C for 15 minutes. Plasma treatments use gas, such as oxygen and argon, to sterilise the sample. In plasma treatments, the hydrophilicity (which helps to enhance cell attachment) is enhanced [100, 101].

Biohybrid scaffolds are a type of scaffold used in tissue engineering that combines synthetic or natural materials with living cells or tissues. These scaffolds are designed to mimic the natural extracellular matrix (ECM) of the tissue being regenerated, providing a supportive environment for cell growth and tissue regeneration. Biohybrid scaffolds can be made from a variety of materials, including synthetic polymers, natural polymers, and ceramics, and can be designed to have specific mechanical and biological properties. Studies have shown that biohybrid scaffolds can facilitate tissue regeneration and integration with native tissue. For example, a study by [102] demonstrated that a biodegradable synthetic polymer microfiber-extracellular matrix hydrogel biohybrid scaffold showed extensive cellular infiltration and integration with native tissue. Another study by [103] showed that a biohybrid gradient hydrogel scaffold facilitated concurrent regeneration of cartilage and subchondral bone in a rat model. There are multiple ways to manufacture biohybrid scaffolds and they are summarised in Fig. 2.6. Fig. 2.6(D), shows the cell-built ECM layer fabrication method which involves the use of living cells to produce an extracellular matrix (ECM) on a scaffold. The ECM produced by the cells provides a natural environment for cell growth and tissue formation. There are several methods for making biohybrids using cell-built ECM layers and they utilise tools, such as bioreactors, to enhance the deposition of extracellular matrix (ECM) on the scaffold, leading to greater and more confluent coverage of the supporting material [104].



Figure 2.6: Methods of Fabricating ECM-Based Biohybrid Materials. (A) Materials are fabricated with interwoven fibers, using either electrospinning, electrospraying, or a combination of the two. (B) Materials are fabricated by blending hydrogel components of ECM-derived proteins and polymer chains. (C) Materials are built using a layering technique, with either a whole tissue or a polymer scaffold as a base. (D) Cell-built ECM layer fabrication, where cells are cultured on a polymer scaffold, and then removed, leaving an ECM layer on the polymer surface. Figure and caption from [6].

#### **Scaffold Material Properties**

**Biocompatibility** To avoid initiating an immune response from the body, a scaffold must be biocompatible. The material of the scaffold must be able to coexist alongside the cells in the body and function properly, without releasing toxic byproducts. If the material is not biocompatible, this could 1) in vitro damage the cells and 2) in vivo cause a rejection which would harm the patient.

**Biodegradability** The need for biodegradability of scaffolds depends on their use. For tissue generation, it would be beneficial for the scaffold to degrade and be replaced by the growing tissue. In implants, such as hip, dental or heart valves, the scaffold needs to remain whole, so to carry out the desired functions. The rate of degradation depends on how and where in the body it is being used. For scaffolds that are biodegradable, over time the scaffold degrades into non-toxic components and is absorbed by the cells. At the same time, the cells grow and take the place of the scaffold, which was acting as a structural template. The rate of degradation is important as if the scaffold degrades at a higher than optimum speed, the cells would lack the necessary structure to migrate, adhere and proliferate. If the scaffold degrades too slowly, this may interrupt cell growth and prolong healing. In [105], the slow degradation rate of scaffolds hindered the growth of new bone. The optimum rate of degradation can also depend on the age of the patient as cellular healing time increases with age [106]. Biodegradability is dependent on the chemical composition of the material, its structure as well as the localised environment in the body. In some cases, the by-products of the scaffold degrading can impact cell action. In the degradation products of silk fibroin scaffolds promoted endothelial cell proliferation [107].

**Porosity** Interconnected porosity is a crucial property for scaffolds to allow for cell penetration, nutrient delivery and waste removal. Pore size is also an important consideration when designing a scaffold [108] so that the pores are large enough for cell spreading. There are common techniques to achieve porosity in scaffolds such as emulsion templating [109], leachable particles [110] and gas foaming technology [111]. Pore size can also affect cell binding, cell attachment and cell migration[112]. Studies have shown that scaffolds with well-interconnected pores can induce cell ingrowth within the scaffold and improve cell density [113, 114].

**Mechanically Relevant** In some cases, where the scaffold is acting in places of a load-bearing tissue type, such as cartilage or bone, the mechanical properties of the scaffold should be consistent with the tissue type at the site of the implant and would function in place of the cartilage for the time it takes for the remodelling to complete.

As discussed earlier, cells are mechano-sensitive so the mechanical properties of the scaffold are important and can influence the cellular response. Scaffold stiffness has been shown to induce and regulate cellular proliferation [115, 116], affect myoblast differentiation [117] and endothelial cell growth [118]. Matching mechanical properties is also important to prevent stress shielding of cells [119]. When administering a force to a scaffold, discontinuities in the scaffold matrix may lead to uneven distribution of force. Cells experience stress shielding from the scaffold, and the scaffold may inhibit force transfer to the cells. These discontinuities are common in scaffolds that are manually fabricated.

With the use of traditional 3D scaffolds, there are a number of drawbacks. The fabrication of such scaffolds is a time-consuming, complex process and is not scalable. Moreover, as the cells are seeded onto an already formed 3D scaffold, there is a heterogeneous distribution of cells in the scaffold. This is caused by the cell's limited ability to penetrate into the scaffolds. Scaffolds produced via emulsion templating have been shown to encourage porosity and improve the mechanical properties of synthetic scaffolds [120]. Therefore scaffolds produced in high internal phase emulsions (HIPEs) form is common in tissue engineering. There are a number of emerging methods of scaffold manufacturing [121] and two of the most popular fabrication techniques are electrospinning and 3D Printing. Multi-media 3D bioprinting methods are a recent approach to generating tissue-like structures.

Over time, the cell-seeded scaffold undergoes changes in its material properties as a result of various factors, including scaffold degradation, cell proliferation, and extracellular matrix (ECM) deposition. In the case of a biodegradable scaffold, its mass diminishes alongside its material properties. Simultaneously, the cells deposit ECM, which contributes to the development of new material properties within the system, such as tensile strength and stiffness. One example where a tissue engineering cellseeded scaffold increased stiffness over time is in the study by [122]. The study investigated the viscoelastic shear properties of hydrogel-based cartilage grafts and found that scaffold stiffness increased with both culturing time and cell density. The study used a hydrogel-based scaffold made of poly(ethylene glycol) diacrylate (PEGDA) and seeded with chondrocytes. The stiffness of the scaffold was measured using a rheometer, and the results showed that the stiffness increased significantly over time as the cells deposited more extracellular matrix. The study concluded that scaffold stiffness is an important factor in the development of functional cartilage tissue, and that scaffold design should take into account the mechanical properties of the scaffold and the cells seeded onto it. In the study by [123], prevascularised microtemplated fibrin scaffolds for cardiac tissue engineering applications showed significant increases in construct stiffness over 10 days in vitro due to extracellular matrix deposition by seeded cells. These studies demonstrate that tissue construct stiffness can change in vitro over time due to various factors, including extracellular matrix deposition, mechanical stimulation, and substrate stiffness.

The findings presented in [57] demonstrate that cells cultured on a PGS-M scaffold exhibited a 50% increase in collagen secretion compared to cells grown on six-well tissue culture plastic plates (control) during a 7-day in vitro experiment. Since collagen is a component of the extracellular matrix (ECM) and contributes to tissue stiffness [51], this increase in collagen production indicates an overall rise in ECM deposition, resulting in an augmented stiffness of the tissue scaffold. However, it is important to note that the stiffness of the PGS-M scaffold itself will diminish due to degradation. To effectively stimulate the cells within a three-dimensional environment, the applied force must be sufficiently large for the cells to sense and respond to it [124]. Therefore, if the global stiffness of the scaffold changes as anticipated, the cells will require a proportional force to detect the same mechanostimulation signal throughout the duration of the experiment.

### 2.1.6 Analysis

Given the small scale of cells and their constituents, the development of cells and tissues cannot be assessed visually by the naked eye. To address this problem there have been various techniques developed to analyse and quantify their development. Picogreen assay directly stains the DNA of cells and is commonly used to determine the cell number in a sample. Picrosirius red assay is a useful assay and allows researchers to quantify the total collagen [125]. Histology is a common technique using a microscope to study the structure of tissue. Samples first need to be fixed, sectioned and stained before histological examination can be conducted and so it is an end-point destructive analysis.

In vitro analysis traditionally focuses on end-point destruction using the techniques described above. In many cases, it would be beneficial to analyse the process of cell growth through an experiment as it is likely to be transient throughout a long-term experiment. It may also be useful to use the same construct developed in an experiment for further experimentation. Advancements in real-time monitoring of cell growth may be able to provide insight into the fundamentals of cell actions and how they impact their environment. Kim et al. [7] report a buoyant e-scaffold able to monitor long-term cell behaviour by measuring the electrical impedance in environments that would be normally unfavourable to electronics, i.e submerged in a cell media (Fig. 2.7a). Other monitoring techniques, such as spectroscopy, imaging, and elastography, are used to monitor cartilage development both in vitro and in vivo [126]. Micro-dialysis has also been used to monitor glucose and lactate as a measure of cellular metabolic activity [127]. Liu et al. [8] demonstrated a microdevice array that has the capability to induce mechanical stimulation and measure the developing stiffness of a construct in situ using integrated on-chip strain sensors (Fig. 2.7b). These non-destructive analysis techniques are a step forward in understanding the cellular behaviour throughout an



entire experiment.

Figure 2.7: Images of implementation of real-time monitoring of tissue constructs. a) Integrated multi-modal arrays of sensing elements within an e-scaffold. Image adapted from [7]. b) Microdevice array with integrated strain sensor. Figure taken from [8].

Biosensors are another method to achieve real-time monitoring of cellular processes. They are defined as a device that uses biological molecules to detect the presence of chemicals [128] and can provide understanding into cellular processes and responses.

## 2.2 Enhancing Engineered Tissue

Engineered tissues can be enhanced in several ways to improve their function and therapeutic potential. The choice of cell source is critical for engineered tissue, as different cell types have varying abilities to proliferate, differentiate, and function in a tissue-specific manner. By selecting and optimizing the cell source, researchers can improve the quality and function of the engineered tissue.

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The scaffold is a crucial component of engineered tissue, providing structural support and a platform for cell attachment and growth. By improving the scaffold design, such as by optimizing its porosity, stiffness, and degradation properties, researchers can improve the mechanical properties of the tissue and its ability to support cell growth and differentiation. In the study by [129], the researchers optimized the porosity, stiffness, and degradation properties of a poly(lactic-co-glycolic acid) (PLGA) scaffold for bone tissue engineering. They found that increasing the porosity of the scaffold improved cell infiltration and nutrient transport while decreasing the stiffness of the scaffold improved cell proliferation and differentiation. Additionally, they found that controlling the degradation rate of the scaffold was important for maintaining the mechanical integrity of the construct over time. The micro-environment of the cells can be tuned to alter the cell action and therefore improve tissue-engineered (TE) constructs. There are different approaches to improving TE construct properties, such as mechanical, chemical and electrical and there is evidence that a combination of environmental factors best represents the optimum biological environment. To include such a range of factors is complex and can be difficult to isolate and understand cellular interactions with each factor so that the environment can be tuned further. Mechanical stress is a critical factor in tissue development and function, as it influences cell behaviour and tissue organization. By applying mechanical stress to the engineered tissue, such as through the use of bioreactors, researchers can improve tissue formation, alignment, and mechanical properties.

Overall, enhancing engineered tissue involves a combination of approaches that optimize the cell source, scaffold design, biochemical signalling, mechanical stress, and vascularisation, and the use of advanced imaging and analysis techniques to evaluate the tissue. By using these approaches, researchers can create tissues with improved function and therapeutic potential for a wide range of applications.

## 2.2.1 Stimulating Tissue

There have been many different approaches to stimulating tissue, the most common include; mechanical, electrical and chemical stimulation.

Stimulating a cell-seeded scaffold can be challenging because it requires replicating the complex environment of living tissues, including the interactions between cells, extracellular matrix, and biochemical signalling pathways. Firstly, cells in living tissues are in close proximity and can communicate with each other through direct contact. In contrast, cells in a scaffold may be more widely dispersed, making it difficult to establish the cell-to-cell contacts that are essential for tissue formation. Secondly, cells in living tissues receive nutrients and growth factors through a network of blood vessels. In a scaffold, however, there may be limited availability of these essential factors, which can limit cell growth and tissue formation. Cells in living tissues are subjected to mechanical stress and strain, which can affect their behaviour and the formation of the tissue. In a scaffold, there may be a lack of mechanical stress, which can make it difficult to create tissues that function properly. In a scaffold, there may be a lack of ECM or an inadequate ECM, which can limit cell attachment and migration and may affect tissue formation. Cells in living tissues also receive biochemical signals from neighbouring cells and the ECM that influence their behaviour and tissue formation. In a scaffold, there may be a lack of appropriate biochemical signals, which can limit cell growth and differentiation.

To address these challenges, researchers are developing new methods to stimulate cell-seeded scaffolds, such as using bioreactors to provide mechanical stress, adding growth factors and other biochemical signals to the scaffold, and developing new biomaterials that can more closely mimic the ECM. By addressing these challenges, researchers hope to create cell-seeded scaffolds that can effectively stimulate tissue formation and ultimately lead to new therapies for a wide range of diseases and conditions.

#### Mechanical Stimulation

The use of mechanical stimulation is already being exploited throughout medicine to promote tissue recovery. In the condition, of Long Gap Oesophageal Atresia (LGOA), surgeons use the Foker technique to apply tension via sutures to the ends of the oesophagus. This promotes tissue growth and once the tissue has grown long enough the two ends of the oesophagus can be sutured together. In rehabilitation after surgery, patients are encouraged to perform load-bearing actions in order to promote their recovery. Another example is where tissue expansion is used to replace tissue by mechanically stretching the tissue over time [130].

For a given stiffness gradient, cells have been shown to progress along it [131] and the differentiation of some stem cells is dependent on their substrate stiffness [132].

Different types of mechanical stimulation are implemented depending on the cell type and desired effect. Compression [133], tension [134] and shear stress [135] are commonly used in tissue engineering to stimulate cells. Compression in tissue is experienced in daily tasks such as walking; where, for example, compression is applied in tissues surrounding the hip, knee and ankle joints [136]. Tensile strain plays a critical role in load-bearing joints [137] and has been shown to improve tensile properties of tissue [138]. Shear stress has been shown to regulate cell proliferation, especially in haemodynamics [135]. The same principle has been applied to other cells, such as chondrocytes [137] and the results include remodelling of the ECM. Given that cells are viscoelastic [139], they must be stimulated within their elastic region to prevent them from deforming.

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A study that outlines a rigorous mechanical stimulation regimen for all cells that produces optimal cell growth, proliferation, maturation etc does not exist as different regimes have been shown to affect cells differently. Whilst still not well understood, there are studies that outline what type of stimulation promotes specific results, a selection is shown in Tab. 2.2. The research studies shown were selected and summarised due to the type of cells used in the study (fibroblasts), the use of stimulation regimes based on sinusoidal stimulation and the use of percentage strain as a stimulation factor. These studies use the same inputs as the robotic bioreactor will use as initial set-up parameters.



**Figure 2.8:** Signal representation of the types of cyclic mechanical stimulation. a) Constant stimulation. b) Intermittent Stimulation, c) cyclic incremental stimulation and d) incremental stimulation.

There are various regimes that have been trialled to stimulate cells and in this report, we will be limiting the scope to sinusoidal stimulation because it provides improved control over elicited activity. Fig. 2.8 outlines four key regimes of sinusoidal stimulation: a) constant, b) intermittent, c) cyclic incremental and d) incremental. Constant stimulation extends a scaffold with the same amplitude in a regular cyclic pattern throughout the entire experiment (Fig. 2.8a) Cyclic stimulation is commonly used to promote cell proliferation in tissue engineering [140, 141, 142]. This type of stimulation has also been shown to induce anisotropy, which is characteristic of native tissue [143]. Kuang's [83] study showed that regardless where the origin of dermal fibroblasts, cycle mechanical strains of 0.1Hz for varying periods increase cell proliferation rates and type I collagen productions. Intermittent stimulation administers cyclic stimulation with rests between sets (Fig. 2.8b) and most closely reflects physiological joint loading. Lohberger [144] applied cyclic stimulation to stimulate human primary rotator cuff fibroblasts. The stimulation regime consisted of 10 seconds of uniaxial sinusoidal mechanical stimulation at a frequency of 0.5Hz and 10% elongation followed by 30 seconds of rest over a 14-day experiment. The results showed a significant increase in the mRNA (messenger Ribonucleic acid) expression and significantly higher amounts of total collagen in the mechanically stimulated fibroblasts. Cyclic incremental stimulation, which is a stimulation where the amplitude of the sinusoidal increases throughout an experiment, was shown in a study to be the best regimen for collagen production compared to cyclic stimulation [134] (Fig. 2.8c). Incremental Stimulation induces forces of increasing amplitudes over the entire experiment (Fig. 2.8d).

In conclusion, extensive research on optimal mechanostimulation protocols reveals that different regimes yield varying outcomes. The specific cell types and environmental factors employed can also influence study results, including cell proliferation capacity and extracellular matrix (ECM) production [144, 134]. However, investigating these variables necessitates time-consuming and lengthy iterative experiments in the pursuit of globally optimal conditions. This is primarily due to the current limitations of tissue engineering tools, which lack adequate sensing and adaptive control methods. Most research groups choose a regime and then compare it to static and report improvements, rather than spend a long time looking at the many variations and variables possible and how they might be optimised.

An important aspect of mechanical stimulation is not long how you stimulate tissue but also how much. For sheet-like scaffolds, this is usually quantified by the percentage of extension. Too little extension and results may present a slow tissue growth rate and too much extensive may plastically deform the cell-seeded scaffold making it more like to tear and fail. Therefore, a balance must be struck between the two. This balance is highly dependent on the material properties of the scaffold itself and the cell type used. A range of 2-30% extension is reported and used for fibroblasts on polymeric scaffolds with the frequency of stimulation ranging from 0.1Hz to 1Hz as shown in Tab. 2.2. It can be concluded that cyclic stimulation has an impact on cell behaviour, and the type of stimulation also has an impact on the type of cell action induced. In [145], an actuated 3D microgel was used to stimulate single cells in 3D environments. Technologies such as these are the first steps in understanding single-cell mechanobiology and being able to exploit them at the multicellular level.

When experimenting with living cells, there must be recognition of change within a system to be able to administer the stimulation regimes truthfully. If a scaffold increases stiffness over time due to ECM deposition and is administered a constant stimulation regime, the actual stimulation applied to the cells decreases over time [8]. A study in [146] reports on how mechanical stimulation needs to be tailored over time to account for increased stiffness of constructs in vitro to elicit predictable and consistent cellular responses.

**Table 2.2:** Current research examples into the effects of various mechanical stimulation regimes. Based on a table in Jeerawan Thanarak PhD Thesis.

Study	Type	Mechanical	Strain	Study	Result
	of cell	Stimulation	& Freq	Length	
Rolin et al.	Human	Cyclic	10%	96 hrs	Increased
[147]	Dermal				differentiation
	Fibroblasts		1 Hz		
Ugolini	Cardiac	Cyclic	2 & 8%	96 hrs	Higher
et al.	Fibroblasts				proliferation
[148]			1 Hz		rate at $2\%$
Lohberger	Rotator	Intermittent	10%	7 &	Increased
et al.	Cuff	Cyclic		14 days	collagen and
[144]	Fibroblasts	(10s stim,	$0.5~\mathrm{Hz}$		ECM proteins
		30s rest)			
Schmidt	Dermal	Constant,	5%	48 hrs	Incremental
et al.	Fibroblasts	Intermittent,		14 days	produced the
[134]		Incremental	$0.5~\mathrm{Hz}$		most collagen
		Cyclic			
Josh et al.	Human	Cyclic	5%	2 & 4	Low freq & strain
[149]	Dermal			weeks	had no effect
	Fibroblasts		0.1-1Hz		High strain was
					damaging
Lee et al.	Fibroblasts	Incremental	7-15%	6 days	Enhancement
[150]		Cyclic			of mechanical
			1 Hz		properties
Manuyakorn	Bronchial	Cyclic	30%	24hrs	Increased
et al.	Fibroblasts			48hrs	collagen
[151]			$0.2~\mathrm{Hz}$	96hrs	production
Kuang et al.	Human	Cyclic	10%	24hrs	Increased
[83]	Dermal			36hrs	proliferative
	Fibroblasts		0.1 Hz	48hrs	rate

# 2.3 Tissue Engineering Tools

In this report, tissue engineering tools are defined as tools used in parallel to the cells, scaffolds and chemicals needed in vitro study. Tissue engineering tools are categorised

into four categories; prediction, production, performance, and preservation, as defined in [20], and are summarised in Tab. 2.3. Prediction tools are ones which use in silico techniques to predict the outcome of experiments or cell action. Production tools aid the manufacturing and/or development of scaffolds and cells. Performance tools used various techniques to capture the functionality of cells through images and non-destructive measurement systems. Preservation tools prolong the life or delay the degradation of samples so that further analysis or use is possible.

Function	Tool	Application	Example Study
Prediction	BioSpice	Primarily geared to represent	Adalsteinsson
		cellular dynamics in 3D fluid	et al.[152]
		mechanical systems	
	Cellular	Represents cellular behavior	Longo et al.[153]
	automata	(migration, differentiation)	
		and dynamics	
	Dynamo	Simulate cell fate & express	Qiu et al.[154]
		behaviours change based on	
		different manipulations	
Production	3D Printing	3D printing of the scaffold or of	Wettergreen
		biopolymers and cells	et al.[155]
			Khalil et al.[156]
	3D	Lithographically defined	Luo et al.[157]
	Patterning	scaffolds	
	Bioreactors	Used to promote cell action	Wang et al.[158]
		through controlled stimulation	Bursac et al.[159]
		methods	[160]
Performance	Multiphoton	3D imaging based on non-	König et al.[161]
	microscopy	linearities in tissue	
	Optical	3D imaging based on local	Xu et al.[162]
	coherence	linearities in tissue where	Fujimoto et al.
	tomography	variations in scattering,	[163]
		refractive index	Mason et al.[164]
	Doppler	Imaging of fluid flow at micron	Yang et al. $[165]$
	OCT	dimensions	Leitgeb et al.[166]
Preservation	vation Cryo- Common approach for		Pegg [167]
	preservation	preservation of cells and tissues	Keros et al. [168]
	Vitrification	Produces solidification	Song et al. $[169]$
		of super cooled liquid into a	Kuleshova et al.
		glass-like state	[170]
	Freeze	Dehydration of samples to	Crowe et al. $[171]$
	Drying	preserve them	

**Table 2.3:** Summary of Tissue Engineering Tools commonly used in the field and key relative studies. Based on the table in [20].

## 2.3.1 Bioreactors

There are various ways to achieve mechanical stimulation with a bioreactor. Commercial mechanical bioreactors are discussed in Tab. 2.4 and a recurring theme in

the disadvantages column can be observed; there is no control or feedback in any of the bioreactors. This means that once the program has been set, the program cannot change unless the experiment is stopped; regardless of how the mechanical properties of the scaffold have changed.

#### **Commercial Bioreactors**



**Figure 2.9:** Commercially available bioreactors. a) Ebers bioreactor [9], b) BioTense bioreactor [10] and c) Ligagen bioreactor [11].

Tissue engineering bioreactors must be manufactured according to Good Manufacturing Practices (GMP) guidelines, which ensure that they are consistently produced to the highest quality standards. International Organization for Standardization (ISO) provides a set of standards for the design, development, and manufacturing of medical devices, including tissue engineering bioreactors [172]. Compliance with ISO standards is required for regulatory approval in many countries. Tissue engineering bioreactors must also undergo biocompatibility testing to ensure that they do not cause adverse reactions or toxicity when in contact with living tissues. Tissue engineering bioreactors must undergo safety and efficacy testing to demonstrate that they are safe and effective for their intended use. This typically involves in vitro and in vivo testing, as well as clinical trials for devices intended for human use. Tissue engineering bioreactors must be approved by regulatory agencies, such as the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA) before they can be marketed and sold for clinical use. Some examples of commercially available bioreactors are shown in Fig. 2.9 and summarised in Tab. 2.4.

Commercially	Advantages	Disadvantages
Available		
Devices		
Ebers TC-3F	User interface is user friendly	No controlled response to
[9]	Multiple chambers	force measurements
	Various types of clamps	Uniaxial stimulation only
	Transparent lid so the scaffold	
	is easily observed	
	Capable of measuring force	
	response of stimulation	
BioTense	Multiple chambers	Circular scaffolds needed
[10]	Able to measure compressive	No monitoring of the overall
	stress	change of the global system
		No control feedback
		Cell Shielding
LigaGen	Multiple chambers can be	Limited thickness of scaffold
[11]	stimulated at once	needed
		No monitoring of the overall
		change of the global system
		No control feedback
		Cell Shielding

**Table 2.4:** Commercially available mechanical bioreactors with their advantages and disadvantages outlined.

#### **Novel Bioreactors**

Researchers have developed their own purpose-built bioreactors to suit their own experiments. It seems that each research group developing their own bioreactor focuses on improving one main aspect of their bioreactor to improve the functionality in comparison to current commercial bioreactors (Tab. 2.4). There has been a wave of bioreactor designs that have the capability to bi-axially load three-dimensional (3D) tissue constructs [173, 174, 12]. Bi-axial stimulation has the advantage as it has similar effects to those found physiologically, where it is unlikely that force is applied in just one direction. A bioreactor developed in [12], uses position control to stimulate a construct via sliding shear and compressive stimulation, bi- or uni-axially. The design is shown in Fig. 2.10a. Axial compression and shear deformations which administer different procedures of strain and frequency are employed by the bioreactor

presented in [173], individually or simultaneously. Lu et al. [175] presented a bioreactor to provide physical stimuli to cardiac fibroblasts. Specifically electrical stimulation, cyclic stretch and fluid perfusion. A miniaturised bioreactor was designed to investigate axon stretch growth with a focus on high accuracy measurements, for which they used a dual-frequency laser interferometer which has high optical resolution [15]. A bioreactor presented in [176] focused on the employment of direct imaging with high magnification during tension stimulation. Other research groups focus on the ability of



Figure 2.10: Novel bioreactors. a) Mechanical stimulation bioreactor system. Image adapted from [12]. b) Bioreactor for Mechanical Stimulation and Real-Time, Nondestructive Evaluation of Engineered Cartilage Tissue. Image adapted from [13]. c) Novel Bioreactor System. Image adapted from [14] d) Miniaturized bioreactor for axon stretch. Image adapted from [15].

the bioreactor to non-destructively monitor the construct in real-time, as shown in Fig. 2.7. Preiss-Bloom et al. [177] and Cook et al. [14] use a load cell to record the real-time force response of a construct whilst applying mechanical stimulation to a cell-seeded scaffold. Preiss-Bloom et al. used this tool to track the transient development of the cell-seeded scaffold and concluded that the mechanical properties of their construct did not develop linearly. A bioreactor presented in [178] combined mechanical stimulation and online micro-computed tomography monitoring to improve the understanding of how mechanical loading affected bone-like matrix deposition. A study,[13], developed a

similarly focused bioreactor, using a load cell to measure the force response and therefore the stiffness of the construct where an ultrasound transducer was also used to evaluate the ECM content. One novel bioreactor focused on mimicking vasculogenesis in the body using ultrasound as the primary method for non-destructive monitoring of the construct [179]. The ultrasound images can be combined to form a 3D image, used to visualise the growing construct. The bioreactors presented in this paper that provide mechanical stimulation to a substrate are, as a whole, hard-bodied and create a rigid boundary between the substrate and actuator. As cells respond to their environment, this rigid interface can affect the cellular response. Bidan et al. [180] applied localised mechanical loading to cells using the magneto-active substrate. Soft magnetic micro-pillars, which were enclosed in a soft elastomer, were used to stimulate cells on a continuous surface to address the problems presented with 'hard' actuation. Although these bioreactors have the capacity to measure the sample non-destructively, to my knowledge, there has been no progression in using this feedback to guide the stimulation reported in the literature.

## 2.4 Robotics

The perception of robots is constantly changing and robotic integration in our daily life is continuously increasing [181]. The definition of a robot is simply "an autonomous machine capable of sensing its environment, carrying out computations to make decisions, and performing actions in the real world" [182]. From this, a huge range of robots have been created.

## 2.4.1 Medical Robotics

In the medical field, robots can improve surgical procedures [183], therapies and rehabilitation [184]. Surgical Robots have been developed to improve the accuracy and precision of surgical procedures. The surgeon can control the robot via a direct telemanipulator or through computer control. The first robot-assisted surgery took place in 1988, where the PUMA 200 was used for selective brain biopsies [185].

## 2.4.2 Soft Robotics

The move towards soft robotics is a fairly recent approach to achieving compliance between living and robotic systems. This compliance has major advantages in the medical field where the physically flexible bodies generally cause less adverse effects, such as fibrosis, and are capable of working in unstructured environments. At a cellular level, a softer environment is considered to better mimic the native environment of soft tissue cells. Soft robotics has various interesting research areas including but not limited to actuation [186] and sensing strategies [187], control methods [188] and finally manufacturing techniques [189].

Polymeric materials are commonly used in soft robotics but are continuously refined and improved with enhanced capability [190, 191]. For a robot to be able to interact with its environment, actuation is key. There have been various approaches to soft actuation and through their non-linear deformations they can be tuned to produce complex motions; such as walking [192] and crawling [193]. Common approaches use pneumatics, hydraulics [194], shape memory alloys [195, 196] and dielectric elastomeric actuators. Stiffness modulation of soft actuators has proven to be a major challenge for researchers. For example, in soft grippers, the actuator must transition from a soft passive state to a stiff active state to apply enough tension to grip the target. Recent studies have emerged to overcome such challenges including using a granular material encased in an elastic membrane [197].

Traditional electronics are typically rigid, and brittle and do not deform well. Therefore, advancing stretchable electronics is crucial to achieving a completely soft robot. Approaches to achieve said electronics include soft conductive polymers [198], conductive fluids into gels [199] and soft micro-fluidic channels [200]. Soft stretch, bending, pressure and force sensors are just a few examples of sensors that have been explored globally to provide localised sensing to soft robots. Soft sensing, electronics and actuation enable truly soft robots with complex control methods to be achieved. However, where compliance and design simplicity are gained within soft robots in comparison to hard-bodied robots, modelling and control of soft robotics becomes significantly more complex and poses a huge challenge [201, 202]. Traditional methods of modelling do not tend to hold up well when dealing with systems that have almost infinite degrees of freedom.

Previously, it is discussed how mechanical stimulation influences cellular activity and how soft robots provide a more compliant platform to interact with the body. This knowledge has been applied to implantable devices which aim to stimulate the cells in vivo. Pneumatic actuation has been used to stimulate tissue to reduce the surrounding fibrotic capsule around the implant [203]. Roche et al. [204] present a soft robotic heart sleeve, which mimics the heart's natural action and material characteristics, to support cardiac activity. Another study [205] introduces a soft device designed to be deployed into the heart to augment ventricular blood ejection due to its small size and soft body. In all of the cases mentioned, revision surgery would be needed to remove the implant. In [206], biodegradable elastomers and Silicon Nanomembranes/Nanoribbons are explored which hydrolyse completely in biofluids or groundwater, presenting the possibility of achieving soft implants that are absorbed by the body when they are no longer needed.

It would be near to impossible to isolate the impact of this stiff interface from the mechanical stimulation. Therefore, using approaches explored in the field of soft robotics to induce this stimulation whilst also enabling real-time monitoring of cellseeded scaffold development is the next logical approach.

## 2.4.3 Robotics in Tissue Engineering

Robots are becoming increasingly important in tissue engineering, providing precise and repeatable techniques for cell and tissue culture, biomaterials handling, and tissue assembly. There have been different approaches to how tissue engineering has been integrated into robotics and vice versa. Some people are exploiting robotics as a tool to advance tissue engineering and others are using tissue engineering to advance robotics. The key difference lies with the overall goal of the robot.

Research has been carried out where biological action drives robot action. By replacing traditional actuators with living cells, the robots can be stimulated electrically or photically. These robots have been labelled as biobots, where the substrate is only living cells, or biohybrid robots, where the substrate is a combination of a polymer and living cells. These types of robotics commonly use heart or skeletal muscle from animals. A biohybrid Jellyfish was developed which is capable of propulsion, where the arms were micropatterned with protein lines to allow cell growth, similar to that found in a jellyfish [207]. A manta-ray-shaped robot was developed that used certain frequencies of light to stimulate heart cells to contract and move the robot [208]. The authors in [209] used a solution of collagen and human dermal fibroblasts, the main components of skin's connective tissue, to coat a robotic finger. The result was not only more human-like skin coverage but also self-healing capabilities.

Bioprinters are specialized robots that can deposit biological materials, such as cells and growth factors, in precise patterns and layers to create 3D structures, mimicking the complex architecture of living tissues [210]. Bioprinting technology allows for the creation of tissues with complex geometries, vascularisation, and cell types, and has the potential to be used for organ and tissue regeneration [211, 212, 213].

Automated cell culture systems use robots to control environmental factors such as temperature, humidity, and nutrient availability for growing and expanding cells. These systems can improve the reproducibility and consistency of cell cultures and reduce the risk of contamination. In [16], the authors present a robotic actuator that applied force-controlled compressive mechanical loading externally to injured skeletal muscle in vivo. The study showed that the mechanical loading reduced interstitial fibrosis and damaged muscle fibres, and also showed larger myofibrils in mechanically loaded tissue. The tetanic force was also shown to be greater when tissue was treated with mechanical loading. Using force control to mediate compressive forces allowed consistent forces to be applied to the tissue during the repair of the injured tissue. Recently, a Humanoid shoulder, shown in Fig. 2.11, was used to provide physiologically relevant stimulation to tissue construct [17]. Although the study did not explore the benefits of using control theory in tissue engineering, this demonstrates the first steps in integrating robotics into tissue engineering. Damian et al. shows, for the first time, the integration of robotics with tissue engineering in vivo [19]. Kanda et al. presented a robotic artificial intelligence system to determine the optimal protocols to grow replacement retina layers, crucial for vision. The robot is seen to not replace human lab work but work alongside, conducting the time-consuming and iterative processes to find the optimal growth conditions [214]. Park et al. presents an organosynthetic dynamic heart model using soft robotic techniques [215]. The conventional bioreactors discussed in Sec. 2.3.1 are hard-bodied and if stiffness does affect cell action, as it has shown in vivo where encapsulation occurs around implants, it is reasonable to conclude that using a stiff apparatus to induce mechanical stimulation will also affect cell action.



Figure 2.11: Examples of robotics in tissue engineering. a) Photograph of robotic soft-interface actuator equipped with a force sensor and demonstration of the actuator positioned toward the injured tibialis anterior (TA) muscle of hindlimb of mouse. Figure and caption from [16]. b) Chamber positioned at the supraspinatus (SS) location on the robotic arm (left) showing the correspondence with the supraspinatus tendon location on a human shoulder (right). Figure and caption from [17].

Surgical robots, such as the da Vinci system, can be used in tissue engineering procedures to provide precise and minimally invasive tissue manipulations. These robots can be used to create tissue flaps, perform tissue dissections, and suture blood vessels. A robotic implant was developed which explored the possibility of regenerating tissue in vivo for conditions where there is 'missing' tissue, such as oesophageal atresia and short bowel syndrome [19]. Plenoptic three-dimensional and near-infrared fluorescent (NIRF) imaging system enabled supervised autonomous soft tissue surgery in open surgery in [18].

Overall, robots are playing an increasingly important role in tissue engineering, enabling researchers to create complex tissues and organs with a high degree of precision and control, ultimately leading to new treatments and therapies for a wide range of diseases and conditions. Tissue assembly robots are used to assemble individual tissue components, such as blood vessels, into complex structures. These robots can also be used to create tissue-engineered constructs that incorporate multiple cell types and biomaterials.

While the use of robotics in tissue engineering has many potential benefits, there are also several challenges that need to be addressed to ensure that these technologies are effective and safe. Tissue engineering often involves the use of multiple technologies, such as biomaterials, cell cultures, and growth factors, which need to be integrated with robotics. Achieving the integration of these different technologies is a complex challenge that requires interdisciplinary expertise.

Tissues in the body are highly complex structures that are composed of multiple cell types, extracellular matrix, and blood vessels, among other components. Mimicking this complexity with robotics is challenging, as it requires precise control over the placement and behaviour of multiple cell types and the ability to create functional blood vessels. The materials used in robotics for tissue engineering must be biocompatible, non-toxic, and able to support cell growth and tissue function. Designing such materials is a significant challenge, as they must be able to maintain their structural integrity over time and interact with surrounding tissues without causing inflammation or other adverse reactions. The use of robotics in tissue engineering raises unique regulatory challenges, as the safety and efficacy of these technologies need to be thoroughly evaluated before they can be used in clinical settings. Establishing appropriate regulatory frameworks for robotics in tissue engineering is critical to ensure patient safety and prevent potential risks. Robotics and related technologies for tissue engineering can be expensive to develop and operate. The high cost of these technologies can limit their accessibility and make it difficult to scale them up for widespread use.

Robotics can be used to automate the screening of different cell types, scaffold materials, and growth factors to identify the most effective combinations for tissue engineering. This approach could significantly reduce the time and cost of the tissue engineering process and increase the efficiency of discovering new materials and techniques. Automated technology can also be used to automate the fabrication of scaffolds, enabling the precise control of scaffold properties such as porosity, stiffness, and degradation rate. This approach could also allow for the creation of complex scaffold geometries that are difficult or impossible to produce manually. Addressing these challenges will require continued investment in research and development, interdisciplinary collaboration, and regulatory oversight. By overcoming these challenges, robotics could play a



Figure 2.12: Robotics in surgery, a) Supervised autonomous robot used in soft tissue surgery. The STAR system integrated NIRF and 3D plenoptic vision, force sensing, submillimeter positioning, and actuated surgical tools. Caption and figure from [18]. b) Robot capable of regenerating oesophageal tissue in vivo. Robot with skin removed to show motor drive system and sensors. Rotation of worm gear causes the lower ring to translate along the body. Caption and figure from [19].

crucial role in advancing tissue engineering and ultimately leading to new therapies for a wide range of diseases and conditions.

# 2.5 Chapter Summary

In this chapter, we have discussed the current scientific understanding of mechanobiology and the tools and techniques commonly used in tissue engineering to enhance engineered tissue. We then went on to discuss robotics as a field and how robotics and tissue engineering have been integrated so far. The future of robotics in tissue engineering is promising, as robotics has the potential to revolutionize the field by providing new tools and approaches to improve tissue engineering outcomes.

What is clear is that this integration is a relatively new research field and is still in its early stages of development. It seems that most research projects that aim to use robotics to enhance tissue engineering have published their pre-cursor papers, presenting the robotic tool design and basic function, but have yet to delve into the profound studies which use complex robotic technology to significantly improve engineered tissue compared to open loop tools commonly used, such as commercial mechanical bioreactors.

The challenges associated with such studies range and require a huge range of skills, experience and an interest in both robotics and tissue engineering. What also may be a limiting factor in the progression in the field is the resources available. Tissue engineering labs would not commonly have access to high-level robotics technology and vice versa. Collaboration between specialities is key for the progression in this field.

# Chapter 3

# Development of the Mechanical and Electrical Design

In this chapter, the development of the robotic bioreactor mechanical design is discussed. The development is split into two distinctive prototypes; the first and second-generation robotic bioreactor. In this report, the mechanical design refers to all the parts of the bioreactor that do not affect the electrical design. This includes, but is not limited to the chamber, clamps, membrane and base. The overall design of the first prototype of the bioreactor is an imitation of the Ebers Bioreactor, initially prototyped by Theo Le Signor, shown in Fig. 3.1 and so this chapter is a continuation of their work. Any reference to the inherited prototype of a robotic bioreactor is in reference to the prototype developed by Theo.

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**Figure 3.1:** Image of the initial prototype of the robotic bioreactor. Taken from Theo Le Signor's Internship Report.

The prototype inherited from previous students used the worm gear and rack, the compression force sensor. I have refined and improved the design of each element and implemented new controllers: force control, position control and stiffness-based position control.

# 3.1 Design Criteria

The design criteria for the mechanical and electrical design of the bioreactor are discussed in this section, with emphasis on why the criteria are crucial to meet and how these criteria are achieved in the design process. The key design criteria are:

- 1. Not Cytotoxic: Cells will be in direct contact with the chamber during in vitro experimentation. A biocompatible material is used to manufacture the chamber, clamps and any components that come into contact with the cells
- 2. **Re-useable**: Bioreactor must be able to support multiple experiments. The chamber is water-tight and can undergo multiple sterilisation processes.
- 3. Maintain Sterile Environment: The environment within the chamber of the bioreactor must be stable. The chamber and clamps are sterilised with 70% isopropanol. The bolts (used to secure the clamps and lid), the lid and the rubber seal are autoclaved.

- 4. Appropriate Size: The bioreactor must fit inside an incubator which has a shelf size of approximately  $65 \times 50$  cm. The size must be cost-efficient to fabricate, a larger device will require more material to produce, increasing the cost.
- 5. Easy to mount and grip a scaffold: Clamps must be designed to efficiently clamp the scaffold to the bioreactor under a class 2 safety cabinet and maintain a grip on the scaffold throughout an experiment.
- 6. Clamp must be allowed to move with minimal resistance: To be able to measure the force exerted on the scaffold, the clamp must be able to freely move with minimal resistance. The method to seal the chamber must accommodate the moving clamp.
- 7. Force exerted on the scaffold accurately measured: Force data is needed to determine the stiffness of the tissue construct during an experiment. A force sensor that can withstand the conditions inside an incubator is used outside of the chamber to measure the force exerted onto the scaffold during an in vitro experiment.
- 8. Accurate extension exerted onto the scaffold: When extending the scaffold, the displacement measurements taken by the robotic bioreactor must be accurate so that the scaffold is being extended to the correct extension percentage as set by the user.

# 3.2 First Generation Robotic Bioreactor

The bioreactor is composed of two main assemblies; 1) the sterilised assembly, areas which will come into contact with cells during an in vitro experiment, and 2) the non-sterilised assembly, parts of the bioreactor which are completely isolated from the cells.

## 3.2.1 Design and Manufacturing of Sterilised Assembly

The sterilised assembly refers to the parts of the bioreactor that need to be sterilised prior to an in vitro experiment to support cell survival. Without sterilisation, bacteria would contaminate and kill the cells.



Figure 3.2: The first generation robotic bioreactor.



Figure 3.3: CAD design of the main components that make up the robotic bioreactor.



**Figure 3.4:** The chamber sealing design. A polyurethane membrane keeps the chamber water-tight and prevents contaminants to enter the sterile chamber, which is kept in place by the rob sealing screw and chamber sealing screw. A small  $Ecoflex^{TM}$  00-50 (Smooth-on Inc.) gasket and rod collar are used to create a better seal at both sealing points. The hole in the lid is to accommodate for the air filter during in vitro experiments.

#### Chamber

The chamber must be a suitable size, to firstly fit inside an incubator and secondly, to house a scaffold, which size is approximately  $10 \text{mm} \times 20 \text{mm} \times 1 \text{mm}$ . Size is also

limited by the Mojo 3D printer capability. The chamber must also be sealed to reduce the risk of contamination of the cells from environmental bacteria and fungi. The chamber of the first-generation bioreactor is fabricated with Acrylonitrile butadiene styrene (ABS) using a fused deposition modelling (FDM) 3D printing technique. This material was chosen as it can tolerate being submerged in alcohol for a short period, crucial for the disinfection of the chamber. Although immerging ABS in alcohol can reduce its tensile strength, this was not highlighted as a risk as no forces are being applied to the chamber. Acetone is added to the chamber for around 20 seconds whilst being swirled to cover all chamber walls. This seals the inner walls of the chamber. This is necessary as printing with FDM means that the walls are not 100% dense and so the liquid could leak through the walls. The acetone, however, if left in the chamber for too long can dissolve the ABS and create larger holes in the chamber walls. To reduce exposure of ABS to acetone, a new chamber is printed for each in vitro experiment so that the chamber will only have minimal exposure to acetone to allow sealing. To allow tolerances for the acetone to seal without melting the wall, each chamber wall was 5mm thick.

The inherited prototype had a chamber that measured,  $130 \text{mm} \times 60 \text{mm} \times 100 \text{mm}$ . This was reduced as it was very bulky and seemed unnecessarily large. The new chamber measured  $116 \text{mm} \times 55 \text{mm} \times 88 \text{mm}$ , with a lid sealing the chamber measuring  $120 \text{mm} \times 105 \text{mm}$  laser cut from a 6mm thick clear acrylic sheet. The acrylic sheet enables the user to see into the chamber whilst maintaining a sterile environment inside the chamber. A moulded ring of PDMS is used to create a seal between the lid and the chamber body. Four bolts and nuts are used at each corner of the chamber to compress the ring to create a seal. During in vitro experiments, clean sterile air needs to be pumped inside the chamber and constantly recycled during the experiment. A 0.22  $\mu m$  filter is used to facilitate gas transfer between an incubator and the cell culture environment. To accommodate this filter, a hole was laser cut in the lid (Fig. 3.4). The sterile filter is added under the class 2 biological cabinet, to reduce the risk of contamination, after the chamber and its counterparts have been sterilized.

There is a hole in the front wall of that chamber that allows the proximal (moving) clamp to be directly connected to the motor and force sensor and move with the motor. This hole creates a pathway for bacteria to enter the chamber and so a barrier is needed to isolate the moving clamp and chamber from the outside environment. It is crucial to isolate the chamber from the external environment to prevent bacterial infection.

The same method of sealing was used as from the inherited prototype design but refined. A membrane is needed to attach to the outside of the chamber using a thread and a counterpart to the thread that tightly screws around the membrane to maintain a strong grip against the junction (Fig. 3.4). This chamber thread and the chamber sealing screw were redesigned to give a smaller clearance between them and to ensure better fit and leak proofing. A small gasket, fabricated from Ecoflex<sup>TM</sup> 00-50 (Smooth-

On Inc.), shown in Fig. 3.4, was added at the end of the chamber sealing screw to ensure a better seal around the chamber hole.

The second part of the membrane is attached to the rod, which the proximal clamp is attached to, using the same threading and screw mechanism. This was also redesigned as the inherited prototype did not have any sealing method, apart from a loosely fitted screw which was not leakproof. Both the screw and thread were designed so that they fit snugly together when tightened. A collar was added to the rod as can be seen in Fig. 3.5. The screw can be tightened to this collar, creating a much better seal. Sealent was also added to prevent further leaks. A waterproof bellow-type seal is created around the chamber whilst allowing the clamp to freely move.

Given that the thread and screw are 3D printed, high friction exists between the two parts making effective tightening of the screw difficult and therefore affecting the ability to achieve a repeatable watertight join. The inherited prototype of this chamber trialled low-density polyurethane as the membrane material. The commercial purpose of low-density polyurethane is to seal food items in containers to keep them fresh over a longer time. It is very flexible and is easily torn, making it difficult to handle and time-consuming to set up.

#### Clamps

The inherited prototype clamps were very large and had an aggressive ridge teething pattern which would cut soft material scaffolds when clamped. These were totally redesigned and reprinted with two ABS clamps, produced by rapid prototyping which featured a less aggressive ridged teething pattern for a secure grip of the scaffold as shown in Fig. 3.5.

Whilst in tension, there must be even force exerted throughout the scaffold as localised force is likely to tear the scaffold. Two hexagon-shaped holes were created in the bottom clamp and in the chamber wall to accommodate two nuts which were positioned and secured with cyanoacrylate adhesive. The top clamps have holes which allow two M3 bolts per clamp to pass through and be threaded into the nuts in the bottom clamp. This enables the clamps to be pressed together, ensuring that the scaffold is secured in place. A guide rod was added to the bottom clamps to allow the clamps to be aligned more easily inside the biosafety cabinet. While one clamp is fixed to the wall of the chamber with bolts (distal clamp), the other one is driven by the external control module along a linear direction to pull and relax the scaffold (proximal clamp). The clamps, not including the rod which is attached to the clamp (proximal), measure  $35\text{mm} \times 38\text{mm} \times 18\text{mm}$ . The moving clamp (proximal) is supported by the chamber floor which is designed with built-in tracks. These tracks inhibit the lateral movement of the clamp, which is likely to damage and tear the scaffold while minimising the friction between the two elements. The clamp (proximal) exits the chamber through a hole in the chamber wall, which creates an entrance for contaminants to enter from the external environment into the chamber. This hole is sealed using the waterproof bellow design discussed earlier. The process of clamping the cell-seeded scaffold is performed under a class 2 biological cabinet. This has contributed significantly to the design of the clamps, so that the clamping can be performed using only sterilized tools, away from the cell-seeded scaffold, to reduce the risk of contamination to the chamber and cell-seeded scaffold.

The clamps bolts and nuts in the inherited prototype were replaced with A4-grade steel nuts and bolts. The previous nuts and bolts all showed significant corrosion and rust after being exposed to a humid environment as so A4 steel was chosen due to its excellent corrosion resistance and high tensile strength.



**Figure 3.5:** The clamp design. CAD drawing of the bioreactor clamps and the mechanism of how the cell-seeded scaffold is secured in place within the bioreactor.

## 3.2.2 Design and Manufacturing of Non-sterilised Assembly

The actuation module is placed outside of the sterile chamber and encompasses electronic components that would not withstand the sterilization process required for the sterilised assembly. The bioreactor is composed of three key electronic components; a microcontroller, a motor and a force sensor. Keeping the electronic components simple, reduces the risk of electrical problems during long in vitro experiments. The inherited prototype used the same motor and force sensor (Honeywell FSS005WNGT) but instead used a Raspberry Pi to control them. An Arduino NANO was chosen to replace the Raspberry Pi as Arduinos are inexpensive, and very good boards for quick programming and circuit prototyping. The overall electronic schematic is summarised in Fig. 3.6.

Earlier in the report, the function and characteristics of modern incubators used for *in vitro* experimentation are discussed. One key aspect, humidity, can damage the electronics. For this reason, the electronics are encased in a sealed waterproof enclosure to protect the electronics. Damaged electronics may give inaccurate results or stop the experiment altogether.



**Figure 3.6:** Simple electronic schematic of the first generation bioreactor.



Figure 3.7: Labelled CAD drawing of the worm gear and rack mechanism.

Motor and gears used to extend the scaffold A 12V DC micro gear motor is used to move the proximal clamp of the bioreactor. The motor has a 1:298 gear ratio, which was chosen for its higher torque output. The decision was made to use a DC motor as opposed to a stepper motor as DC motors are inherently closed loop and a stepper motor is inherently open loop. A closed-loop component allows for the implementation of position control.

To monitor the displacement of the scaffold clamped inside the bioreactor chamber, a magnetic encoder, attached to an extended shaft on the DC motor, counts the number of turns the magnet makes which is proportional to the number of turns the motor makes. The distance travelled along the rack can be calculated which allows the motor to be controlled to follow a target displacement trajectory. The motor is used to actuate the worm-gear mechanism. The rotation motion is converted to translational motion with a rack designed with the same parameters. This means that with one  $2\pi$  rotation, the rack is theoretically moving 1.95mm. This actuation chain implies an important ratio r between one motor rotation (sensed by the encoder) and an actual rack translation. The theoretical movement of the rack when one step of the magnetic encoder is detected is 550nm.

The motor's rotational motion must be converted into linear motion. This is achieved with a worm gear, with a pitch of 1.95mm and rack design with the same parameters (Fig. 3.7). The gears were designed to try to minimise backlash to improve displacement accuracy. Backlash is the play or movement between two mechanical components that are supposed to be in contact with each other and is common in gears and motors. Manufacturing tolerances, wear and tear and elastic deformation are the common causes of backlash. Some amount of clearance is necessary to prevent the teeth from binding or jamming. However, excessive backlash can result in a delay or lag in power transmission, as well as an increase in noise and vibration. In this case, the backlash would be most likely caused by the inaccuracies of 3D printing.

#### Force Transduction Mechanism

A compression force sensor (Honeywell FSS005WNGT), with a 0 to 5N sensing range with a sensitivity of 7.2mV, was used in combination with an amplifier to measure the force exerted onto the scaffold. The gain of an amplifier is important because it determines the degree to which the input signal is amplified by the amplifier. A resistor was plugged into the component to amplify the force signal. The force sensor (Honeywell FSS005WNGT) was soldered onto a prototype board and glued with Cyanoacrylate adhesion onto the hook mechanism to secure it in place. The force sensor (Honeywell FSS005WNGT) was sampled every 25ms using a timer interrupt. The force sensor (Honeywell FSS005WNGT) was calibrated using a pulley and calibrated weights and then plotted to give two linear calibration regressions of y = 383.84x - 22.16 and y = 23.792x + 493.44 (Fig. 3.9). At around 0.2N, there is a sensor noise where multiple force sensor readings were taken when measuring the same weight. The mode of these points is 50 which falls on the calibration line. Any noise in the sensors is reduced by taking a rolling average in the code for the bioreactor.



Figure 3.8: Force transduction mechanism to use a compression force sensor (Honeywell FSS005WNGT) to sense tensile forces. a) Labelled image and illustration of the force transduction mechanism. b) Labelled illustration showing how the hook compresses the force sensor (Honeywell FSS005WNGT) when the clamps in the chamber are pulled apart and releases the tension on the force sensor (Honeywell FSS005WNGT) when the motor pushes the clamps together.
A mechanism was designed to accommodate a compression force sensor (Honeywell FSS005WNGT) that can detect the amount of force applied to the scaffold through each pulling cycle. This sensor was soldered to a protoboard and then placed and glued between the hook of the teethed rack and the rod of the proximal clamp. Fig. 3.8 illustrates how when the motor pushes the clamps together, the hook attached to the motor releases compressive forces on the force sensor (Honeywell FSS005WNGT) and then when the motor pulls the clamps apart, the hooks attached to the motor push on the force sensor (Honeywell FSS005WNGT) to sense tensile forces. The mechanism relies on a small amount of movement in the mechanism. Although this 'gap' is crucial for the force transduction mechanism to work, it adversely affects the displacement accuracy of the motor. Rails in the base allow the compression sensor to move in one axis, mitigating the risk of sensor error due to misalignment of the sensor and hook.

Calibrated weights were used to calibrate the compression force sensor (Honeywell FSS005WNGT). A chamber was 3D printed with PLA with an inbuilt pulley mechanism to enable calibration of the sensor horizontally with the chamber membrane assembled. This setup ensures calibration is as accurate as possible in the bioreactor in vitro set-up. Weight was converted to Newtons using the equation  $F = w \times 0.0098$ . The mean and standard deviation were computed and plotted in Fig. 3.9. A calibration curve was then computed and plotted. Although there is a clear trend, The average standard deviation is 0.2518V and there is a large overlap of standard deviations. This high standard deviation is higher than expected from the data sheet performance characterisation. This difference can be explained by the way the sensor is being used to measure force in the bioreactor, rather than issues with the sensor itself.

Since the sensor is the connection point between the upstream section of the actuation chain (i.e. the motor) and the rod of the clamp that is pulling the scaffold, the force sensed is approximately equal to the actual force applied onto the scaffold. Friction forces do exist between the moving elements of the bioreactor and only contribute to a minor difference between the actual force applied to the scaffolds and the sensed force. In this design, the sensor can only capture the force as the clamps are pulled apart. When the clamps move back together the force sensor (Honeywell FSS005WNGT) is completely unloaded, as shown in Fig. 3.8, and therefore is no useful force data.

To address this issue a second design of the force transduction mechanism was designed and trialled to capture the loading and unloading of the scaffold. The same concept was used, a hook compresses the force sensor (Honeywell FSS005WNGT and Force Sensor B in Fig. A.1) when the clamps in the chamber are pulled apart. A second force sensor ((Honeywell FSS005WNGT, Force Sensor A in Fig. A.1) was added behind the hook, as shown in Fig. A.1. Both sensor signals were amplified and then the sensors were calibrated with calibrated weights. This dual force transduction mechanism was trialled and presented in Fig. A.2. In the preliminary testing of the two-sensor design, the force sensors did not follow the stepped trajectory of the position of the



**Figure 3.9:** Compression force sensor (Honeywell FSS005WNGT) calibration plot achieved using calibrated weights. Average measured sensor readings and their standard deviations are plotted in blue and a calibration curve is plotted in red. (n=6)

clamps and sensors remained in tension due to friction forces. The setup was difficult to make repeatable and the hook mechanism is working in the 'stiction' region, so is highly non-linear. To measure the force, there is a high reliance on the scaffold to 'pull' the clamps back together. This design was no longer considered after these failed preliminary trials.

#### Base

The base was designed to support and align the three main components of the bioreactor; the force transduction mechanism, the motor and gears, the clamps and the chamber. The base provides stability to the robot and prevents the components from moving. Any misalignment of components will not only affect the action of the bioreactor but also its ability to sense movement and force accurately. The base of the robotic bioreactor features tracks to avoid lateral deflections during the linear motion of the rod. This is important as the scaffold is delicate and any sudden force may damage the scaffold and affect the results of the experiment. The base also ensures the line of transmission stays linear which is vital for accurate force readings. The bioreactor is calibrated for a specific setup, any change in that setup would detrimentally affect the bioreactor's function.

The bioreactor and base fit into an area measuring  $343 \text{mm} \times 110 \text{mm} \times 63 \text{mm}$ , so will fit in most conventional incubators that provide the required environmental conditions

for cell survival.

As this robotic bioreactor is a prototype, the base was also designed so that there is easy access to the robot's components, this allows for easier maintenance and repairs. In summary, a well-designed base is critical for ensuring the stability, support and precision of a robotic bioreactor.

### 3.2.3 Challenges Presented

From working with the first-generation bioreactor there were some areas highlighted for improvement during testing and use. The main areas were as follows:

- The chamber from the first prototype was larger than necessary. It used more material to form the chamber and as there was a large volume to fill, more cell culture media is needed to culture one cell-seeded scaffold and therefore increased unnecessary costs.
- Using acetone to seal the chamber from leaking through the walls was difficult to control. Once the acetone was left in the chamber for too long, it created holes in the chamber walls that allowed the liquid to leak out as shown in Fig. 3.10.
- The chamber membrane was not robust enough for long-term experiments. The polyurethane used as a membrane was easily torn by the screw and therefore created holes in the membrane for the liquid to leak out of. The screw attached to the rod, outside the chamber, allowed the liquid to pool in the membrane outside of the chamber when the chamber was being moved.
- The force transduction mechanism was difficult to set up repeatably. If incorrectly constructed, the friction present in the hook mechanism applies force to the force sensor (Honeywell FSS005WNGT) when no force is being applied to the scaffold. It was also easy for the force transduction mechanism to become misaligned. When a force is redirected, its direction changes because it is now being applied in a new direction. The magnitude of the force may or may not change, depending on the circumstances. In this case, as the forces sensed are relatively low, any noise and alterations will affect the measured force. Trackers were added to reduce misalignment but this increases the friction between the moving parts. This alignment also applies to the clamps moving within the chamber.



Areas where cell media has leaked through



• Significant gear backlash existed between the worm gear and translational rack Gears require precise dimensions and tolerances to function properly. The worm gear and translational rack were both 3D printed. 3D printers may not always produce parts with the required accuracy, especially if the printer is not well-calibrated or if the filament used is not of high quality. The surface finish of a gear is also important because it affects how well the teeth mesh together. 3D printing can produce rough surfaces that can negatively affect the performance of gears.

Proper sensor placement is critical to obtaining accurate and reliable data that can be used to monitor and control the conditions within a system. The optimal placement of sensors may depend on various factors, such as the size and shape of the system, the nature of the process or reaction being monitored, and the type of sensor being used. It is important to carefully consider the placement of sensors in a system to ensure that the data collected is representative of the system as a whole and can be used to optimize the performance of the system.

## 3.3 Second Generation Robotic Bioreactor

The aim of the second-generation robotic bioreactor was to address some of the problems presented in the first generation that could not be overcome without major design changes. The focus on the second generation is making the bioreactor modular. Modular design is important for prototyping because it allows for flexibility and scalability in the development process. A modular design approach involves breaking down a complex system into smaller, more manageable components, which can be easily modified or replaced without affecting the rest of the system. When it comes to prototyping, this approach enables quick iteration and experimentation with different designs and configurations, without having to rebuild the entire system from scratch every time. By building and testing individual modules separately, designers can identify and address issues early in the development process, before they become more costly and difficult to fix. The modular design also makes it easier to customise and adapt a prototype to meet specific needs or requirements. For example, if a particular module or component needs to be upgraded or replaced with a newer version, it can be done without affecting the rest of the system.

### 3.3.1 Design and Manufacturing of Sterilised Assembly

#### Chamber and Sealing

The inner dimensions of the chamber are  $76\text{mm} \times 51\text{mm} \times 50\text{mm}$  and the outer dimensions of the chamber  $102\text{mm} \times 83\text{mm} \times 55\text{mm}$ . This reduces the base area, therefore, reducing the volume of cell media needed to submerge the cell-seeded scaffold and supply nutrients to the cells.

The new chamber was printed from Dental SG resin (Formlabs) on Form 3 3D printer, which is biocompatible [216]. The resin is autoclavable but after a few cycles becomes brittle so areas that are easily cracked. Areas of the chamber that are vulnerable to cracking, for example, where the lid is attached to the chamber, were reinforced with more material.

The chamber sealing method was changed to a gasket-based design as shown in Fig. 3.12. The chamber was redesigned with a built-in flange (Chamber Flange in Fig. 3.12) with six holes equally spaced around the flange. A gasket was created from Mold Star<sup>TM</sup> 15 SLOW (Smooth-On Inc.) 15 SLOW, with a thickness of 5mm, as this gave the best results for sealing and did not have any adverse effects to being submerged in alcohol for disinfection. A second flange was laser cut from a 3mm thick acrylic sheet (Acrylic Flange in Fig. 3.12). A polypropylene (PP) membrane is used to isolate the



**Figure 3.11:** The second-generation bioreactor. The top image is the top view of the second-generation bioreactor and the bottom image is a side view image of the second-generation bioreactor.

chamber and allow free movement of the rod and proximal clamp. Polypropylene is autoclavable and therefore will not deform during sterilisation. A comparison of the membrane material options is summarised in Tab. 3.1. The membrane is shaped and heat-sealed to produce a tubular shape, with a small hole at one end and a skirt at the opposite end. The PP membrane is sealed outside the chamber by pressing the acrylic flange onto the chamber Mold Star<sup>TM</sup> 15 SLOW (Smooth-On Inc.) gasket. The PP membrane skirt is placed between the chamber Mold Star<sup>TM</sup> 15 SLOW (Smooth-On Inc.) gasket and the chamber flange. Six screws and nuts compress the parts together and two acrylic washers spread the load of the screws across the entire flange. This is necessary as the dental resin cracks easily with local forces. The screws were bolted using "two-finger tightness", and then each screw was tightened half a turn at a time to enable even distribution of the compression forces and minimise the risk of the chamber flange cracking. Once the chamber Mold Star<sup>TM</sup> (Smooth-On Inc.) gasket was visually bulging evenly around the flange, the chamber was filled with water to inspect leakage. If a leak was detected, the screws were further tightened.

Material	Thickness	Temperature	Common Purpose	
		Range		
Polypropylene (PP)	$404 \mu m$	134°C	Packaging	
Polyurethane (PU)	$12.7\mu m$	93°C	Food Preservation	
Polyvinyl chloride	$200\mu m$	$105^{\circ}\mathrm{C}$	Industrial applications	
(PVC)			(belting, seals, gaskets)	
Linear Low Density	$150 \mu \mathrm{m}$	$60^{\circ}\mathrm{C}$	Films and packaging	
Polyethylene				
(LLDPE)				

 Table 3.1: Material options for the bioreactor chamber membrane.

The rod sealing was moved to the inside of the chamber to reduce the risk of liquid pooling in the membrane outside the chamber walls and leaking out. To make a seal around the rod, a thread, printed as part of the rod, allows a rod screw to press the acrylic washer against a Mold Star<sup>TM</sup> 15 SLOW (Smooth-On Inc.) gasket and rod flange. The rod is printed with Dental SG Resin (Formlabs) as part of it is exposed to the cells within the chamber. A rod screw was chosen to compress the gasket as it reduces the maximum cross-sectional area of the rod so that more of the rod can pass through the chamber pipe, so to enable greater travel of the clamps.

#### Clamps

The basic design of the clamps resembles the clamps presented in the first prototype, with a few changes to improve performance and ease of use. The clamp gripping mechanism was changed from continuous rows of teeth to an array of nodules to improve the distribution of the clamping force across the clamped part of the scaffold.

### 3.3.2 Design and Manufacturing of Non-sterilised Assembly

#### Base

With the previous base design, the fixed motor position did not cause any issues as the stroke length with the worm gear and rack was 80mm. However, the linear actuator used in the second prototype has a stroke length of 10mm which limits the flexibility of set-up configurations that can be achieved by the robotic bioreactor. To allow the



**Figure 3.12:** Exploded illustrated image of the gasket sealing design for the second bioreactor prototype. Orange parts are the parts 3D printed with Dental Resin SG (Formlabs), Cyan parts are fabricated from Mold Star<sup>TM</sup> 15 SLOW (Smooth-On Inc.), and white parts are laser cut from an acrylic sheet. The grey shaded area represents the Polypropylene membrane.

position of the linear actuator to be altered depending on the parameters such as scaffold length and percentage displacement. The base is printed via Fused Deposition Modeling (FDM) with PLA, with a 75% infill. This gave a good balance between strength and cost. Its design ensured maximum flexibility whilst the prototype was being refined. It is composed of two slots that span the entire base. These act as slotted bolt holes to allow secure components, but also enable flexibility when setting up the bioreactor and swapping out components. This base design also supports the aim to make the second-generation bioreactor modular.

#### Microcontroller

The same as the previous prototype, Arduino NANO is used as the microcontroller for the bioreactor. The sampling rate of the Arduino captured all the information necessary in previous trials.

#### Motor

For the second iteration of the robotic bioreactor, the DC motor and encoder were replaced with a linear actuator (Actuonix) with an in-built potentiometer to remove some of the play in the system and reduce the displacement error. Shielded cables are used to connect the motor to the Arduino to reduce sensor electrical interference. The motor is attached to the base using bolts and nuts so can be easily detached and replaced if the motor is faulty.

#### Force transduction

The force sensor was changed from the compression force sensor to a tensile force sensor (Richmond Industries 900 Series low capacity load cell (RI 900)), with a 0-5N force range, which replaces the force transduction hook mechanism in the previous design. The sensor has two in-built threads at either end of the sensor which enables easy integration into the bioreactor design with 3D printed attachments allowing one end to be attached to the rod of the proximal clamp and the other end attached to the moving end of the motor. The removal of the hook mechanism should reduce the noise in the system and improve displacement accuracy. A 0-5V module was also added to the new force sensor (RI 900). A voltage module connected to a sensor typically serves to amplify and/or condition the signal output from the sensor. In this case, the voltage module performs signal conditioning functions, such as filtering out noise and interference and adjusting the signal voltage levels to match the input requirements of Arduino Nano.



**Figure 3.13:** The tensile force sensor (RI 900) low-friction support module.

The tensile force sensor (RI 900) used in the second iteration is heavier than the previous sensor and requires support to keep the sensor from buckling the translational line. Bearings are used as wheels to support the weight of the force sensor (RI 900), shown in Fig. 3.13, whilst also enabling low friction movement of the sensor during movement of the motor and therefore the displacement of the tissue scaffold. The wheels are suspended by a frame and fixed to the base using nuts and bolts through the slotted bolt-fixing holes. The slotted bolt-fixing holes allow adjustments to the set-up to be made without the need for reprinting 3D-printed parts.

The tensile force sensor (RI 900) was connected to the motor using a 3D printed part in PLA and is shown in Fig. 3.14a) and Fig. 3.14b). The tensile force sensor (RI

900) has fixed in-built threads which allow for connectors to be bolted on. A U-shaped part was printed and bolted onto the tensile force sensor (RI 900). A through-bold was used to connect the tip of the linear actuator to the connector and spacers ensure that the linear actuator tip remains in the centre of the connection. The other side of the force sensor (RI 900) is connected to the clamp rod and is shown in Fig. 3.14c) and Fig. 3.14d). Similarly to the motor-force sensor connection, a U-shaped part was 3D printed in PLA and bolted onto the force sensor (RI 900) using the fixed in-built force sensor threads. A second part is 3D printed from PLA and is secured to the U shape connection with a through-bolt. The part has an incut section and a bolt, which the clamp rod can attach and be secure.

a) Top view of motor to force sensor connector



Spacers

Through bolt

c) Top view of force sensor to clamp rod connector b) Side view of motor to force sensor connector



 d) Side view of force sensor to clamp rod connector



**Figure 3.14:** Force sensor (RI 900) connectors. a) Top view of the motor to force sensor connector. b) Side view of the motor to force sensor connector. c) Top view of the force sensor to clamp rod connector. d) Side view of the force sensor to clamp rod connector.

A force sensor (RI 900) cable support was 3D printed from PLA and is shown in Fig. 3.11. This reduces the risk of altering the set-up during an experiment as when the cable was moved, it affected the angle of the force sensor (RI 900), which in turn affected the force sensor's accuracy.



**Figure 3.15:** Tensile force sensor (Richmond Industries 900 Series low capacity load cell) calibration plot achieved using calibrated weights. Mean and standard deviation of the sensor readings are plotted in blue.

Fig. 3.15 shows the calibration line achieved using the tensile force sensor (RI 900) with calibrated weights and a pulley system. A chamber with an in-built pulley was printed in PLA via FDM (Prusa) to allow the chamber membrane to be assembled during calibration.

Weight was converted to Newtons using the equation  $F = w \times 0.0098$ . The line shows a linear relationship between the analogue force reading and the force (N).

# 3.4 Chapter Summary

This chapter presents the mechanical and electrical design of the two prototypes developed in the project. The second prototype changes were made with informed improvements from the experimentation of the first. The main changes in the second prototype were the chamber membrane design, changing the motor to a linear actuator, and changing the force sensor to a tensile force sensor (RI 900) to improve the performance and versatility of the robotic bioreactor. The second prototype of the robotic bioreactor can be adapted to be used for compression stimulation as opposed to tension stimulation with design changes to the chamber and clamps only. All presumptions of design improvements will be explored further in Ch. 5.

# Chapter 4

# Development of the Robotic Bioreactor Control Strategies

In this chapter, the development of the robotic bioreactor control methods is presented and discussed. While typical bioreactors have predefined open-loop control, due to changes that occur in the cell-seeded scaffold, it is imperative to have a cue of the evolution of the scaffold and be able to adjust the control signals accordingly. Various stimulation regimes are implemented by the bioreactor to show the versatility of the bioreactor and methods to improve the initial scaffolding protocol are presented and discussed. The first-generation bioreactor was used to develop the control protocols and generate the plots in this chapter. In later chapters, these same foundation control algorithms were adapted and implemented in the second-generation bioreactor.

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# 4.1 Design Criteria

The design criteria for control systems remain similar across most systems and are as follows:

- 1. **Stability**: The control system must be stable, meaning that it should not oscillate or diverge over time. Stability is essential for maintaining the desired output and preventing damage to the system.
- 2. Accuracy: The control system should be able to achieve the desired output accurately. This means that the system should be able to compensate for disturbances, noise, and other factors that can affect the output.
- 3. **Responsiveness**: The control system should be responsive, meaning that it should be able to quickly adjust to changes in the input or output. This is especially important in systems that require fast response times, such as in robotics or manufacturing.
- 4. **Robustness**: The control system should be able to operate reliably even in the presence of uncertainty, such as variations in the system parameters or disturbances in the environment.
- 5. **Performance**: The control system should be able to achieve the desired performance metrics, such as speed, accuracy, or energy efficiency.
- 6. **Complexity**: The control system should be designed to be as simple as possible, while still achieving the desired performance. This can help reduce costs and make the system easier to understand and maintain.
- 7. **Cost**: The control system should be designed to be cost-effective, taking into account the cost of hardware, software, and maintenance.

## 4.2 Robotic Bioreactor Control Methods

In this chapter, two different control types are introduced and discussed, position control and force control. Position control uses the motor sensor feedback to control the stimulation of the cell-seeded scaffold. A position control signal is provided to the bioreactor. Force control uses a force control signal. The clamp moves until the desired force is reached and so the force sensor measurements control the position of the clamps and therefore the stimulation. A PD controller was designed and tuned accordingly for the position control and the force control and was implemented in an Arduino Nano. The force sensor readings were filtered with a moving average filter in post-processing. A phantom scaffold, fabricated from Ecoflex<sup>TM</sup> 00-30 (Smooth-On Inc.), measuring  $30\text{mm} \times 10\text{mm} \times 4\text{mm}$  was used in both the position control experiments, and a scaffold fabricated from Ecoflex<sup>TM</sup> 00-50 (Smooth-On Inc.), measuring  $30\text{mm} \times 10\text{mm} \times 4\text{mm}$  was used in both the position control experiments. To achieve the phantom scaffolds, A and B parts for the Ecoflex<sup>TM</sup> were mixed in a mixer (Thinly ARE-250) in equal parts, degassed for three minutes and then poured into PLA moulds. They were left overnight to cure at room temperature.

The initial position of the proximal clamp was remotely changed, with buttons to control the motor, to ensure that the scaffold was pre-tensioned the same amount in each trial. The clamps were moved until the force sensor measured a force, i.e. once the force measured above 0N. A range of sinusoidal wave frequencies, from 0.1Hz to 1Hz, is used to strain and stimulate cells. A frequency of 0.25Hz is used in all plots which fall within this range to ensure that the bioreactor is capable of achieving the same stimulation as the literature. As discussed in the literature, an extension applied to the scaffold, around 7 - 10% of its original length, shows to stimulate cells and induce cell action.

### 4.2.1 Position Control

For the position control, a sinusoidal target waveform is set with a frequency of 0.25Hz, an amplitude of 3mm and an offset of 3mm to achieve a maximum displacement of 6mm and minimum displacement of 0mm. A scaffold was fabricated from Ecoflex<sup>TM</sup> 00-30 (Smooth-On Inc), measuring 20mm  $\times$  10mm  $\times$  1mm, and clamped to the scaffold. The initial position of the clamps was remotely changed with buttons to control the motor to ensure that the scaffold was pre-tensioned the same amount and zeroed. The data obtained from the trial was plotted in Fig. 4.1.

In Fig. 4.1, the desired trajectory of the position control is shown as a sinusoidal waveform and can be visually compared with the force and displacement measurements taken from the bioreactor. The displacement measurements taken from the Arduino follow the reference signal very well with minimal variation. Following the peaks in the force sensor reading, a sharp drop in force is shown. This is expected as the force sensor is only being compressed when the clamp is pulling the scaffold in tension. When the clamp direction is reversed, no compression forces are acting on the force sensor. The secondary peaks that occur at approximately 7 and 11 seconds are likely a result of the combination of the viscoelastic behaviour of the phantom scaffold and the sensitivity of the force sensor.



**Figure 4.1:** Displacement control of the scaffold using first prototype of robotic bioreactor (DC motor and compression force sensor). (Top) The position control signal (red) shows the desired trajectory of the clamp displacement compared to the actual displacement of the clamps (blue). (Bottom) Force sensor readings during position control experiment. Positive displacement represents pulling the scaffold apart.

### 4.2.2 Force Control

For force control, a sinusoidal waveform with an amplitude of 0.5N and an offset of 0.6N at a frequency of 0.25Hz was implemented. A scaffold fabricated from  $\text{Ecoflex}^{\text{TM}}$  00-50 (Smooth-On Inc.), measuring 20mm × 10mm × 1mm, was clamped in the chamber and the data obtained from the robotic bioreactor was plotted in Fig. 4.2.

The force measurements taken from the bioreactor follow the general trend of the reference signal but with some variation in the data. Similarly to the force measurement in Fig. 4.1, as the tension is released on the scaffold and the clamps move back together and there are no compression forces acting on the force sensor. This, however, has more significant effects when implementing force control as the sharp drop in force significantly increases the error between the reference force and the measured forces. This results in the motor moving to increase the force, as shown in the small secondary peaks in the displacement plot (Fig. 4.1). These secondary peaks occur just after 2 seconds, 6 seconds, 10 seconds and 14 seconds. This motor movement creates jerky clamp movement which affects the stimulation applied to the scaffold and could also damage a delicate tissue construct.



**Figure 4.2:** Force control of the scaffold. (Top) The force control signal (red) shows the desired trajectory of the force measured by the force sensor compared to the actual force readings (blue). (Bottom) Displacement of the clamps from their original position during the force control experiment.

## 4.3 Capability of different stimulation regimes

Many different stimulation regimes are possible with the robotic bioreactor. In Fig. 4.1 and Fig. 4.2 cyclic stimulation is already demonstrated. Intermittent stimulation is demonstrated in Fig. 4.3 at a frequency of 0.25Hz and 3mm amplitude with a 3mm offset, bringing the maximum displacement to 6mm. After three cycles of the sinusoidal waveform, the stimulation reaches its resting period (10 seconds) and then resumes stimulation. The displacement measurements in Fig. 4.3, follow the trajectory well. The first displacement measurement peak in each stimulation set doesn't quite reach the 6mm displacement as the reference signal does not start at 0 and instead starts at 3mm. Therefore the first force measurement peak in each stimulation set also doesn't quite reach passed 1N, whereas the other peaks surpass 1N. This can be easily fixed in further experiments by starting the stimulation reference signal at 0mm. However, the plot still shows how the capability of the bioreactor to perform an intermittent stimulation regime.

Incremental stimulation is demonstrated in Fig. 4.4, where the amplitude and offset of the target sinusoidal force signal increase over time which is achieved by increasing the displacement of the scaffold until the desired force is achieved.

A sinusoidal target waveform is set, with a frequency of 0.25Hz, an increasing ampli-



**Figure 4.3:** Position control with intermittent cyclic stimulation. (Top) The position control signal (red) shows the desired trajectory of the displacement measured by the encoder compared to the actual encoder readings (blue). (Bottom) Displacement of the clamps from their original position during the force control experiment.

tude starting at 0.4N and an offset that also increases at the same rate at the amplitude to keep the reference signal above 0N. Frequencies of 0.1Hz to 1Hz have been used to stimulate fibroblasts in previous studies, so we chose 0.25Hz for our reference signal to sit within this range [83, 217, 134].

A desired force trajectory compared to the actual force measured by the force sensor is shown. There is a general trend of the force sensor reading following the trajectory and the position of the rack in response to the force control. The amplitude of the target force signal increases over time which is achieved by increasing the displacement of the scaffold until the desired force is achieved. This regime would need to be highly tuned so that the stimulation does not exceed that of the mechanical strength of the cell-seeded scaffold in such a way that tears it. An alternative way to avoid such a tear would be to characterise the maximum force that the scaffold can safely withstand and use force control to make sure the maximum force is not exceeded. This would also have to be tuned to the changing mechanical properties of the cell-seeded scaffold so that the maximum force is calculated depending on not only the scaffold type, but also the type of cells seeded and the duration of the experiment.



**Figure 4.4:** Force control of the scaffold. (Top) The force control signal (red) shows the desired trajectory of the force measured by the force sensor compared to the actual force readings (blue). (Bottom) Displacement of the clamps from their original position during the force control experiment.

## 4.4 Scaffold Tensioning Protocol

One issue that persisted with the bioreactor was obtaining the correct tensioning prior to the experiment. Too much tension and the scaffold would rip, too little tension and the cells would not receive the target train strain. This problem extends past this project and to any scaffold stimulation tool aiming to apply a specific strain. To address this issue, a force tensioning sequence was designed as described in Fig. A.3. To summarise, the bioreactor executes its sinusoidal stimulation regime, and after a set period, three stiffness measurements are taken consecutively via a series of ramp steps, where the clamps move apart in steps, where they pause for 2 seconds between each step. The force is averaged over the three stiffness measurements at each step, and if the average force is above 0N, no force tension occurs. If there is no force above 0N, a ramp signal is executed and the clamps move 5% of the maximum displacement that the bioreactor administers (input strain (%)) at a constant speed. The clamps stop for force seconds and the force is averaged over five seconds. If the new average is above 0N, we continue with the sinusoidal stimulation. If the force is still 0N, the force tensioning ramp signal is repeated another two times. Once we have executed three force tensioning ramps, and there is still no readable force, the experiment is stopped, the time is logged and the user is alerted. The reason to limit the number of force tensioning ramps is that if the scaffold is tensioned a total of 10% of the maximum displacement during normal stimulation (strain (%)) and there is no readable force, it

is likely that the scaffold has ripped or there is a problem with the sensor. Stopping the experiment and alerting the user increasing the chance that the experiment can be corrected with user intervention and continued with the scaffold intact.

# 4.5 Chapter Summary

Implementing two different controllers into the bioreactor demonstrates its versatility as a tool. The position control appeared to be more robust than the force control as there was less noise in the encoder readings than there was for the force sensor. This was predicted as force sensors measure the force applied to a surface or object, and this force can be influenced by a variety of factors, including vibrations, temperature changes, and electromagnetic interference. Additionally, force sensors often have a relatively low signal-to-noise ratio, which means that the measurement signal is weak compared to the level of noise present in the system. This can result in a relatively high level of noise in the measurements obtained from force sensors. On the other hand, motor encoders measure the rotational position and velocity of a motor shaft. Because the rotation of the motor is a relatively smooth and continuous process, motor encoders are generally less susceptible to sources of noise than force sensors. Additionally, motor encoders often have higher signal-to-noise ratios than force sensors, resulting in more accurate and less noisy measurements.

Force control, however, is a novel approach to induce mechanostimulation onto a cellseeded scaffold. Both control methods can be used to achieve stiffness-based control which may be a step closer to achieving optimum mechanostimulation for a cell-seeded scaffold where the force applied to the evolving scaffold is truly constant throughout an experiment.

# Chapter 5

# Performance Characterisation of the Robotic Bioreactor Prototypes

In this chapter, the performance of both prototypes of the bioreactor is characterised and compared. Three performance areas have been identified as key to the success of the bioreactor; 1) displacement accuracy, 2) force measurement accuracy and 3) the bioreactor's ability to hold liquid without leaks. The two prototypes are compared in the three performance areas and evaluated.

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# 5.1 Displacement Accuracy

The displacement accuracy of the bioreactor is arguably the most important performance area of the bioreactor. The base function of a uniaxial tensile mechanical bioreactor is to stimulate a tissue construct to a percentage extension. Accurate displacement measurement is crucial for the reliability and validity of the results obtained from tensile bioreactors for several reasons. Tensile bioreactors are commonly used to determine the mechanical properties of biological tissues, such as their stiffness, strength, and elasticity. Accurate measurement of tissue deformation is necessary for accurate characterization of these properties. Inaccurate measurements can lead to erroneous conclusions and misinterpretation of the tissue's mechanical properties. Accurate displacement measurement is important for ensuring that results obtained from different tests can be compared meaningfully. If the bioreactor measures displacement inaccurately, the results obtained from different tests will not be directly comparable, and any observed differences could be due to measurement errors rather than true differences in the tissue's properties. In addition, tissue engineering involves the fabrication of biological tissues using cells and scaffolds. Accurate displacement measurement is essential for optimizing the mechanical properties of engineered tissues. Inaccurate measurement can result in sub-optimal tissue engineering, leading to poor tissue functionality and reduced efficacy.

In summary, accurate displacement measurement is essential for the reliable characterization of the mechanical properties of biological tissues, the comparison of results obtained from different tests, and the optimization of tissue engineering. Therefore, displacement accuracy is a critical factor to consider when designing, using, and interpreting data from tensile bioreactors.

The typical synthetic materials used as scaffolds are selected to mimic native material properties. They are typically soft, slightly elastic and have a low tensile strength. The size of such tissue constructs is limited to the size of the equipment in the lab as it needs to fit inside incubators and under safety cabinets. In experiments carried out in the commercially available Ebers bioreactor, the scaffolds need to fit inside the chamber that has internal dimensions of  $38 \text{mm} \times 65 \text{mm} \times 32 \text{mm}$ .

As discussed in the literature review, typically constructs are extended 2 - 10% of their original length during mechanical stimulation, a maximum of 2mm extension in this study. If the actual displacement was significantly different to that measured by the microcontroller, due to mechanical noise in the system or sensor inaccuracy, the construct could be either under and over stimulated. Research has shown that the stimulation parameters are important and have an impact on cell action, which would affect the results of the study. In these experiments, displacement error is defined as the difference between the measured sensor reading, as transduced by the microcontroller



Figure 5.1: Experimental set-up for webcam tracking to quantify the displacement error of the bioreactor. a) Illustration of the webcam experimental set-up. b) Image of the experimental set-up. c) Top-view image of the robotic bioreactor chamber with the x and y axis defined. d) Still capture of the video retrieved by the webcam to be tracked on Tracker with the x and y axis defined.

compared to the webcam tracking.

In this section, the DC motor from the first prototype and the linear actuator from the second prototype were directly compared. Although labelled as just the DC motor, the mechanical parts such as the worm gear and rack used in conjunction with the DC motor to enable clamp movement will affect the displacement accuracy of the motor. To compare the performance of the two motors, a webcam (Spedal 920PRO) was set up directly above the bioreactor chamber, as shown in Fig. 5.1.

#### 5.1.1 Short-term displacement accuracy displacement

To test the displacement accuracy over a short-term experiment, a webcam (Spedal 920PRO) was used to record a 3-minute video of the proximal (moving) clamp whilst the bioreactor executed the stimulation regime. A sinusoidal waveform was used as the control signal, the same as the stimulation method used in vitro. The stimulation regimes were varied with regard to frequency (0.5Hz and 1Hz) and maximum displacement (0.5mm, 1mm and 2mm). Each stimulation regime was repeated six times for both the DC motor and encoder in the first bioreactor prototype and the linear actuator in the second bioreactor prototype without a scaffold. To enable a direct comparison of the motors, the same compression force sensor and hook mechanism (Fig. 3.8) was used. Therefore, any difference in the two motors will be a direct result of the action of the motor. A sticker was added to the clamp to enable better tracking. A decision to remove the scaffold from the system when conducting this experiment was made, as different scaffolds with different stiffness would affect this result. Therefore, by removing it, we remove the possibility that the results obtained here are only relative to one scaffold with specific material properties. A video tracking programme (Tracker) was used to track the movement of the clamp and measure its position in each frame. The position of the proximal clamp was plotted and the displacement peaks were isolated in MATLAB. The peaks indicate the maximum displacement reached for each sinusoidal wave. The relative errors were calculated from these peaks. The mean and standard deviation of these peaks across the six trials was computed and plotted as boxplots (Fig. 5.2). Relative error was used as it takes into consideration the different lengths of the displacement. The relative error is calculated as:

$$\delta = \left| \frac{v_A - v_E}{v_E} \right| \cdot 100\% \tag{5.1}$$

Fig. 5.2 shows how the two position accuracy between the two motors compares in the x-axis. This is the axis in which the stimulation is exerted. The position tracked by the webcam (Spedal 920PRO) can be compared to the position transduced by the microcontroller, but also to each other. The dotted line is the 0% relative error. The average relative error is also outlined in Tab. 5.1 For the linear actuator at a frequency of 0.5Hz, the standard deviation was 0.04mm, and for 1Hz, it was 0.03mm.

To assess any significant differences between the DC motor and the Linear Actuator concerning frequency and maximum displacement, a one-way ANOVA was conducted with a significance level of p=0.05. The analysis revealed that the only non-statistically different results were observed at 1Hz with a maximum displacement of 2mm. This finding suggests that the choice of motor indeed influences displacement errors. It's crucial to note that when two sets of results are considered statistically different,  $p_j0.05$  is that the chance that these two sets of values are from the same population is only 0.05 or less.



Figure 5.2: Comparison of displacement accuracy of the DC Motor and Linear Actuator without a scaffold clamped in the x-axis. The webeam tracked the movement of the chamber for sinusoidal frequencies 0.5Hz and 1Hz, with a maximum displacement of 0.5mm, 1mm, and 2mm. The measured displacement peaks were isolated and relative errors were plotted as boxplots. \* shows a significant difference between the two groups (p=0.05). (N=3).

However, a different perspective emerges when examining the average relative errors detailed in Tab. 5.1. Despite the statistical differences noted earlier, the average relative errors for both the DC motor and the linear actuator were remarkably similar across all trials and conditions, with 10% and 9%, respectively. Notably, the results for the linear actuator exhibited significantly smaller standard deviations, highlighting the consistency of the measurements. It's essential to recognize that statistical differences can arise even when means are similar if the variance or standard deviation between the two datasets differs.

The average error within the trials was different. The average error within trials refers to the average of the errors observed across different trials or conditions within the same experimental setup. While the average relative errors for the DC motor and the linear actuator were similar, there could still be differences in the average errors

Frequency	Displacement	Average		Average	
(Hz)	(mm)	Relative		Standard	
		Error (%)		Deviation (mm)	
		DC	$\mathbf{L}\mathbf{A}$	DC	LA
0.5	0.5	21.2	12.8	0.11	0.03
	1	9.2	10.9	0.03	0.02
	2	4.1	7.5	0.22	0.05
1	0.5	12.9	5.5	0.01	0.01
	1	4.2	11.7	0.04	0.04
	2	8.3	7.7	0.40	0.05

**Table 5.1:** Table presenting the average relative errors for both the DC motor (DC) and the linear actuator (LA) for 0.5Hz and 1Hz, 0.5mm, 1mm and 2mm, in the x-axis, as shown in Fig. 5.2. (p=0.05)

within trials. Even if the average relative errors are similar, the specific errors observed in each trial might vary. For example, in the DC motor, there could be trials with higher errors compensating for trials with lower errors, resulting in similar averages but different individual trial errors. The average relative error is a measure of accuracy, while the standard deviation reflects the precision or consistency of the measurements. If the linear actuator exhibits lower standard deviations, it suggests more consistent measurements within trials. On the other hand, the DC motor might have higher variability from trial to trial, contributing to differences in average errors within trials. The motors might react differently to specific conditions or frequencies, leading to variations in trial errors. For instance, certain conditions at a given frequency might favour one motor over the other, impacting the average error within those trials. There could be interaction effects between frequency and displacement that influence the performance of the motors differently in each trial. The combination of these factors might lead to variations in average errors within trials. In addition, differences in the experimental setup or environmental conditions during each trial could contribute to variations in measurements. Inconsistencies in external factors may affect the performance of the motors differently in different trials. Random fluctuations or noise in the system could also contribute to differences in average errors within trials. These fluctuations might not follow a specific pattern but can impact individual trials randomly.

In summary, even if the average relative errors are similar, the differences in average errors within trials could be attributed to trial-to-trial variability, sensitivity to specific conditions, or other factors that affect the precision and consistency of the motors' performance. Understanding the underlying causes of these differences may require further investigation into the specific conditions and parameters of the experimental setup.

The average standard deviation across all displacements for the DC motor and en-

coder at a frequency of 0.5Hz was 0.12mm and at 1Hz was 0.15mm. Whereas, the average standard deviation across all displacements for the DC motor and encoder at a frequency of 0.5Hz and 1Hz was 0.03mm. The higher standard deviation for the DC motor and encoder can be explained by the fact that 3D-printed gears will never be as precise or durable as injection-moulded or machined parts. 3D printing can produce a rough surface finish, which can cause increased friction and wear on gear teeth. This can lead to premature failure of the gear system. The accuracy and dimensional stability of 3D-printed gears can be lower than those produced using traditional manufacturing methods. This can result in gears that are not precise enough for use in high-performance applications and create noise and backlash in the system which could explain the larger standard deviation. The precision of 3D-printed gears can be influenced by several factors inherent to the 3D printing process. While significant advancements have been made in improving the precision of 3D-printed parts, there are still certain challenges that may impact the overall accuracy and precision of intricate components like gears. Most 3D printing processes build objects layer by layer. This layered construction can result in visible layer lines and may introduce slight variations in the dimensions of the printed part. While these variations are often minimal, they can affect the overall precision of intricate features such as gear teeth. The resolution and tolerance of the 3D printer can play a crucial role. Some desktop 3D printers, especially those in consumer-grade categories, may have limitations in achieving extremely fine details or tight tolerances. High-precision 3D printers with fine resolutions are available but may be more expensive and less commonly accessible. The choice of printing material can impact precision. Some 3D printing filaments may have slight variations in material properties, and factors like shrinkage during cooling can affect the final dimensions of the printed part. Post-processing steps, such as sanding or smoothing, may be required to achieve the desired surface finish and precision. However, these steps may not always be as effective or consistent as the finishing processes used in traditional manufacturing methods. 3D-printed parts may exhibit anisotropic properties, meaning their precision can vary in different directions. This can be a consideration for applications where uniform precision in all dimensions is crucial. While these factors may contribute to perceived limitations in precision, it's important to note that ongoing advancements in 3D printing technologies and materials are continually addressing these challenges. High-precision 3D printers, advanced materials, and optimized printing techniques are becoming more accessible, enabling the production of increasingly precise components, including gears. From these experiments, it can be concluded that the linear actuator shows consistently improved accuracy across all parameter combinations and so will be the motor chosen to continue experiments with. The experiments conducted not only provide insights into the performance of the linear actuator but also inherently characterise the accuracy of the DC system. The characterisation of the DC system's accuracy is crucial, and it might not have been predicted without a detailed experimental analysis. The results highlight the DC system's behaviour under different frequencies and displacements, revealing patterns of average relative errors and standard deviations. These findings contribute valuable

information about the accuracy, precision, and consistency of the DC motor, shedding light on its performance characteristics in real-world conditions that might not have been anticipated beforehand.



Figure 5.3: Comparison of displacement accuracy of the DC Motor and Linear Actuator without a scaffold clamped in the y-axis. Webcam tracked the movement of the chamber for sinusoidal frequencies 0.5Hzand 1Hz, with a maximum displacement of 0.5mm, 1mm, and 2mm. The measured displacement peaks were isolated and plotted as boxplots. (N = 6).

For the same set of data, the displacement in the y-axis was also tracked and so had the same conditions as the x-axis measurements. The measured displacement peaks in the y-axis were isolated in MATLAB and the data was plotted as boxplots in Fig. 5.3. As the expected value is zero, the relative error cannot be used. This is because the formula for relative error involves dividing the absolute error by the expected value. When the expected value is zero, dividing by zero is undefined and mathematically impossible.

Both the DC motor and linear actuator show variation in the y-axis.

Each condition for both the linear actuator and the DC motor showed some move-

Frequency	Displacement	Average Y		Average	
(Hz)	(mm)	Displacement		Standard	
		Peak (mm)		Deviation (mm)	
		DC	LA	DC	LA
0.5	0.5	0.24	0.23	0.10	0.03
	1	0.36	0.42	0.30	0.32
	2	0.29	0.20	0.24	0.12
1	0.5	0.13	0.19	0.12	0.10
	1	0.20	0.34	0.15	0.22
	2	0.26	0.56	0.17	0.69

**Table 5.2:** Table presenting the average relative errors for both the DC motor (DC) and the linear actuator (LA) for 0.5Hz and 1Hz, 0.5mm, 1mm and 2mm in the y-axis, as shown in Fig. 5.3.

ment in the y-axis. The results are summarised in Tab. 5.2. The average y-displacement peaks were highest at 0.36mm for the DC motor at 0.5Hz frequency and 1mm displacement. For linear actuators, average y-displacement peaks were highest at 0.56mm for 1Hz frequency and 2mm displacement. All of the y-displacement peaks are smaller than 0.6mm. Results that are too small can be unreliable because they may be affected by random variations or errors that can occur during the measurement process. When the results are too small, it becomes more difficult to distinguish between the true signal and the noise in the data, which can lead to inaccuracies and false conclusions. The accuracy of the webcam-tracked displacement measurements is limited by the image quality of the webcam and the tracking software. Therefore, no conclusions can be drawn from these results other than that neither motor produces significant y-axis displacements.

As the robotic bioreactor is only designed to provide uni-axial stimulation, there is no programmed movement in the y direction, which is supported by the results of this experiment. Any displacement in the y-axis may produce undefined shear stress to a cell-seeded scaffold which would affect the cell activity during an in vitro experiment.

### 5.1.2 Three-day displacement accuracy experiment

In vitro experiments with mechanical bioreactors can last from one week onwards. A three-day-long experiment was conducted with the second prototype of the robotic bioreactor with a similar experimental set-up as Fig. 5.1 but with another webcam (Spedal 920PRO) positioned above the linear actuator. This enables the tracking of both the clamps and linear actuator to enable a comparison of movement at the motor with movement at the clamps and whether the movement is lost downstream of the

motor. This will also help clarify whether the displacement measurement taken at the motor position can be used to estimate movement at the clamp position. A 1Hz sinusoidal waveform was used as the control signal with a maximum displacement of 2mm. These parameters were chosen as they can represent the more extreme parameters that a user may choose for an in vitro experiment. Both the clamp and the motor were tracked over three days to measure whether there was any drift in measurement accuracy with the linear actuator in the system over an extended period. Due to the processing limitations of the tracking software, 1 minute of video was recorded every three hours. In this experiment, an  $Ecoflex^{TM}$  00-30 (Smooth-On Inc.) phantom scaffold measuring 20mm × 10mm × 1mm was clamped in the chamber and so a force is sensed on extension.



**Figure 5.4:** Window of data from the three-day experiment The top plot is the force reading from the force sensor plotted against time. The second plot is the linear actuator potentiometer reading. The third plot is the webcam-tracked motor position. The fourth plot is the data obtained from the webcam positioned above the chamber which was used to track the moving clamp in the chamber. Time should be interpreted in hours, i.e. 17.094 hours is equivalent to 17 hours, 5 minutes and 38 seconds.

Fig. 5.4 shows a window from the three-day experiment at around 17 hours into the experiment. The force, potentiometer from the linear actuator, the webcam-tracked motor and the webcam-tracked chamber are plotted. They all follow the same sinusoidal trajectory showing that the webcam is tracking the clamps to the correct



trajectory.

**Figure 5.5:** The results from 1Hz frequency and 2mm maximum displacement 30-second experiment using the linear actuator is replotted from Fig. 5.2 and the three-day experiment where the linear actuator and the moving clamp were both recorded with webcams with the same parameters (1Hz frequency and 2mm maximum displacement). The mean and standard deviations of the peak displacements are plotted. The dotted line on both plots represents the average reading from the potentiometer on the linear actuator. The minimum value of the motor displacement in the chamber (3 day run) is aligned with the bottom of the box.

To compare whether there is any change to the displacement accuracy over a longer experiment, the previously plotted 6 trials of the 30-second experiment are compared to a single trial of the three-day experiment. Both trials use the same input parameters for the sinusoidal control signal, (1Hz frequency and a maximum displacement of 2mm). The peak displacement results from Fig. 5.2 and the three-day experiment are shown in Fig. 5.5. As shown in Fig. 5.5, when firstly comparing the chamber data from the 30-second experiment and the three-day run experiment, a slightly larger standard deviation of 0.09mm with numerous outliers is shown in the three-day experiment compared to a standard deviation of 0.05mm for the thirty-second trial. The mean peak displacement for the 30-second experiment is 1.86mm and for the three-day run is 1.97mm, which translates to an 8% and 2% relative error respectively. These small differences in mean and standard deviations could be explained by the accuracy of the experimental set-up itself, or by other external influences. As the three-day experiment was conducted continuously, the lighting of the lab would have changed slightly due to daylight and artificial light changing as other lab users turned on and off light

switches, which could have influenced the quality of the video quality. As both standard deviations are less than 5%, they are considered to be small and not significant so it can be concluded that the length of the experiment does not influence the displacement accuracy.

Next, the motor and the chamber for the three-day experiment can be compared directly to determine whether there is a difference between the displacement measured at the motor and that measured at the chamber, but more specifically the moving clamp. This could help determine whether there is any mechanical noise downstream of the motor that could lead to displacement loss or disruption at the clamp position. The displacement at the clamp position is very important as it is the displacement that the tissue construct would be exposed to during an in vitro experiment. The mean displacement peak was 2.06mm for the motor and as stated previously, 1.97mm for the chamber, which translated to a 3% and 2% relative error respectively. The standard deviation at the motor position is 0.07mm and at the chamber, the standard deviation is 0.09mm. These small differences are low and not significant, however as two experiments with the same experimental setup and conditions are being compared and the results are very similar, it can be concluded that there is no significant difference between the displacement at the motor and the clamp position. Therefore the displacement measured by the motor can be used as the displacement measurement at the clamp position. The difference in the results could be due to a few different reasons, one is that motors often produce mechanical vibrations as they operate. These vibrations can introduce noise and interfere with the accuracy of movement measurements taken near the motor. When you move further away from the motor, the vibrations tend to dampen, resulting in a more stable measurement environment with reduced interference. Another theory is that the two different webcams, although the same model, could have been focused slightly differently and resulted in inconsistencies in image quality or format between the two webcams may affect the performance of the image processing algorithms, potentially introducing pixelation artefacts or inaccuracies in the displacement measurements.

Considering the absence of the scaffold in the experimental setup, it's essential to recognize the potential impact of its addition on the accuracy of the displacement achieved by the linear actuator, particularly in a bioreactor context. The introduction of a scaffold, especially one with high elasticity, can alter the dynamics of the system, akin to adding a spring. This addition may influence the force-displacement relationship, requiring careful consideration of material properties. The resistance imposed by the scaffold can affect the accuracy of displacement by introducing additional forces that the actuator must overcome. Material properties of the scaffold, such as stiffness and non-linearity, play a pivotal role in determining the extent of this impact. Non-linearities in the force-displacement relationship, including hysteresis, can further complicate the accurate control of the actuator system, particularly if assumptions of linearity are made. To address these challenges and ensure displacement accuracy in the presence of a scaffold, a comprehensive characterization of the actuator system's behaviour with the added scaffold becomes imperative. This involves understanding the force-displacement relationship, identifying any non-linearities or hysteresis, and potentially calibrating the actuator system or adjusting control signals to compensate for the introduced resistance.

As a consideration for future experiments, it might be worthwhile to explore whether introducing a damping element into the system could help mitigate any undesirable effects arising from the interaction between the actuator and the scaffold. Damping elements can contribute to stabilizing the system dynamics and improving control precision, potentially enhancing the accuracy of the achieved displacement, especially in the presence of complex mechanical interactions introduced by the scaffold. This avenue could be explored in subsequent studies to optimize the performance of the actuator system in the context of the specific experimental conditions.

## 5.2 Chamber Isolation Testing

During an in vitro experiment, the chamber will house the cell-seeded scaffold and the cell media which provides nourishment to the cells during the experiment. The chamber must be sealed to; 1) prevent cell media from leaking out which would affect how much nutrients are available to the cell and 2) prevent any bacteria from entering the chamber which would potentially kill the cells. In addition, tissue engineering bioreactor chambers often apply mechanical loading to the growing tissues to promote tissue development and maturation. Isolating the chamber from the surrounding environment allows for better control of the mechanical loading conditions, which is essential for optimizing the tissue growth and maturation process. In this experiment, the two methods of sealing the chamber present in the first and second-generation robotic bioreactors are compared on their ability to isolate the chamber from the outside environment, both through the walls and through the membranes designed and discussed in Ch. 3. The first method of sealing is the threaded screw method present in the first-generation bioreactor and the second method of sealing is the gasket-based design presented in the second-generation bioreactor. To ensure that the membrane designs were being directly compared, both chambers were fabricated from dental SG resin and both membranes were fabricated from polypropylene (PP).

In the first experiment, 50ml of water was added to the control (a plastic beaker), the first-generation membrane (threaded screw design) chamber and finally the second-generation membrane (gasket-based design) chamber. The initial volume of the water was recorded and the control and chambers were left for twenty-four hours. The two chambers were placed on trays/containers to catch any leaks as shown in Fig. 5.6. 50ml is approximately the volume of cell media added during an in vitro experiment and so

was selected for the starting volume of water. Water is used as the liquid substitute for cell media due to the high cost of cell media. No lids were attached to the control beaker or the chambers.



**Figure 5.6:** The experimental set-up to test chamber leakage over 24 hours.



Figure 5.7: Comparison of the two methods of sealing the chamber around the rod when 50ml of water was added to the chamber and left for 24 hours. The blue bar represents the water volume at the start of the experiment and the green bar represents the volume of water at the end of the experiment. Sample N=3.

This experiment was repeated three times and the results are plotted in Fig. 5.7. For each trial, the membrane was entirely deconstructed and reconstructed as would be the case for each in vitro trial. The average control end volume, after twenty-four hours, was 45ml with a standard deviation of 2ml across the three trials. The first-generation (threaded screw) membrane's average end volume was 22ml with a standard deviation of 3ml. The second-generation (gasket-based) membrane's average end volume was 44ml with a standard deviation of 12ml. The lower end volume for the control can be explained by evaporation as no lid was added to the beaker. However, as there was a similar area exposed to the environment for the control and both chambers, it is assumed that evaporation rates were consistent across the beaker and both chambers for each trial. The high standard deviation for the first-generation membrane (threaded screw) end volume can be explained due to the difficulty in constructing the membrane. Due to the threaded design, it is difficult to ensure that the membrane is evenly distributed and it tends to bunch up when the screw is tightened in the chamber. This bunching also affects the amount you can tighten the screw, which in turn affects the effectiveness of the membrane to seal the chamber. It is also worth noting that the chamber hole is approximately halfway up the chamber wall. Once the water falls below the hole, the membrane no longer affects the volume retained in the

chamber. So the volume left in the chamber with the threaded screw membrane design at the end of the trials cannot be attributed to the effectiveness of the membrane but instead, that the water has fallen below the level that it can escape from. The second-generation (gasket-based) membrane shows a much smaller standard deviation which can be attributed to the fact that it is easy to construct the membrane and makes the membrane performance repeatable. A one-way ANOVA was performed on the trials and it showed how the end volumes from the first-generation (threaded screw) membrane were significantly different to both the control and the second-generation (gasket-based) design. There was no significant difference between the control and the second-generation (gasket-based) design. From this experiment, it can be concluded that the second-generation gasket-based design is a more effective sealing method than the first-generation design.

As discussed above, during an in vitro experiment approximately 50ml of cell media would be added to the chamber. However, this could change due to many different factors. During an experiment, the cell media is changed so that cells have sufficient nutrients available to them. To reduce interruptions in the experiment, a larger volume of cell media may be added to the chamber so that the cells have nutrients available over a longer period of time. Therefore it is important to test the membrane to its extremes and identify any weak points that need to be addressed.

For this experiment, only the second-generation gasket-based design is tested as the previous experiment showed that it performed better than the first-generation design. The membrane was isolated into two parts; 1) the inner rod membrane attachment (Fig. 5.8a), and 2) the chamber membrane attachment (Fig. 5.8b). For the inner rod, the membrane was flipped inside out, held vertically and filled with water, as shown in Fig. 5.8a. To clarify in Fig. 5.8a, the top of the rod would be where the clamps attach to and the bottom would be where the force sensor attaches to. The membrane was held up for a few minutes above a blue paper towel to easily visualise any leaks. After three minutes, there was no sign of leakage from the membrane and so the experiment was stopped.

For the chamber, the PP membrane provides a continuous barrier around the chamber hole, ie. there was no hole in the PP membrane that would normally be created to accommodate the rod. The water was added until the water level passed the top of the chamber hole and the chamber was left on a blue paper towel. After three minutes there were no signs of leakage from the membrane and so the experiment was stopped. Although this experiment was very simple, it allows quick evaluation as to whether there is any serious fault with the membrane design.


**Figure 5.8:** The deconstructed chamber membrane during extreme volume testing. a) Isolated inner rod membrane attachment and b) isolated chamber membrane attachment.

From this set of experiments, it is clear that whilst the second-generation chamber is more reliable over multiple trials, there is still a small amount of variation when constructing the membrane. For that reason, before any in vitro experiment, the membrane should be tested at extreme water levels to ensure that the chamber membrane is effective in isolating the cells from the outside environment.

It is more likely any leaks that occur would be through the PP membrane. The decision to leave the chapter static during the experiment instead of whilst the rod is moving is because any movement in the rod is a maximum of around 2mm, which is not enough movement to cause mechanical stress to the membrane and gaskets, which could cause further leaks. By filling the chamber past the point it would normally be filled in a vitro experiment, we push the limits of the system. Testing in this extreme case allows evaluation of the performance and capabilities of a chamber under challenging conditions. Having water surrounding the membrane, which would not be the case in vitro, there is increased pressure on the membrane. Water is much denser than air. Density refers to the amount of mass per unit volume. The molecules in water are packed closely together, while the molecules in air are more spread out. Since water molecules are more tightly packed, they exert more force on a given area compared to the molecules in the air. In summary, filling something with water increases the pressure more than filling it with air due to water's higher density and incompressibility.

# 5.3 Force Measurement Accuracy

Force measurement accuracy is important for tissue engineering bioreactors that have force-measuring capabilities. Tissue engineering bioreactors are commonly used to characterize the mechanical properties of biological tissues, such as their stiffness, strength, and elasticity. Accurate measurement of the forces applied to the tissues is essential for the accurate characterization of these properties. Tissue engineering involves the fabrication of biological tissues using cells and scaffolds. The mechanical properties of the tissues are critical for their functionality and effectiveness. Accurate force measurement is essential for optimizing the mechanical properties of engineered tissues, as it allows researchers to monitor and control the forces applied to the growing tissues. Numerical models are often used to simulate the behaviour of biological tissues under different loading conditions [218, 219, 220]. Accurate force measurement is necessary for validating these models, as it allows researchers to compare the predicted forces with the actual forces applied to the tissues. Similar to displacement accuracy as discussed above, accurate force measurements are also important for ensuring that results obtained from different tests can be compared meaningfully. If the force measurement is inaccurate, the results obtained from different tests will not be directly comparable, and any observed differences could be due to measurement errors rather than true differences in the tissue's properties.

Overall, accurate force measurement is critical for the reliable characterization of the mechanical properties of biological tissues, the optimization of tissue engineering, the validation of numerical models, and the comparability of results obtained from different tests. Therefore, force measurement accuracy is an essential factor to consider when designing, using, and interpreting data from tissue engineering bioreactors.

Measuring force accurately is crucial for the successful function of the robotic bioreactor as it will be used to directly calculate the stiffness of the tissue construct. This stiffness calculation would be used for the control feedback. If the force, and therefore the stiffness, are not accurate, the stiffness-based control could apply inappropriate stimulation. For example, if the stiffness measured by the robotic bioreactor was larger than the true stiffness of the cell-seeded scaffold, the robotic bioreactor would change the stimulation to suit a stiffer construct and would potentially damage the construct.



**Figure 5.9:** *a)* First-generation compression force sensor (Honeywell FSS005WNGT). b) Second-generation tensile force sensor (RI 900).

The first-generation force sensor refers to the compression force sensor (Honeywell FSS005WNGT), used in the first-generation robotic bioreactor, as shown in Fig. 5.9a). The second-generation force sensor refers to the tensile force sensor (RI 900), used in the second-generation robotic bioreactor, as shown in Fig. 5.9b) and introduced in Ch. 3.

## 5.3.1 Tensile Testing of phantom scaffolds

To determine whether the phantom scaffolds fabricated from silicone could be used in place of scaffolds, the stress-strain relationship of the phantom scaffolds was obtained with a uniaxial tensile test on an electric static testing instrument (MX2, IMADA). Two samples of Ecoflex (Ecoflex<sup>TM</sup> 00-10 and Ecoflex<sup>TM</sup> 00-30 (Smooth-On Inc.) were prepared, measuring  $30 \text{mm} \times 10 \text{mm} \times 4 \text{mm}$  and one sample of PGS-M, measuring  $10 \text{mm} \times 25 \text{mm} \times 1 \text{mm}$ . Both Ecoflex<sup>TM</sup> samples were pulled at a constant speed of 1 mm/sec and the PGS-M sample was pulled at a rate of 0.7mm/sec. The Ecoflex<sup>TM</sup> samples were pulled to 15mm displacement to ensure we test within the elastic region of the Ecoflex<sup>TM</sup>. The PGS-M, which is used as a scaffold in vitro experimentation, was pulled to 3.5mm displacement, beyond which its breaking point is reached. Each of the Ecoflex<sup>TM</sup> samples was tested three times. The experiment was then repeated with the first-generation bioreactor. One trial was conducted with the PGS-M sample as these samples are not readily available. Force measurements for both the IMADA and bioreactor were filtered using a rolling average in post-processing and plotted in Fig. 5.10.

The results show that the Ecoflex<sup>TM</sup> 00-10 and Ecoflex<sup>TM</sup> 00-30 (Smooth-On Inc.) fall in the same force range as PGS-M. Although the Ecoflex<sup>TM</sup> samples do not quite match the material properties of PGS-M, they are similar enough to justify their use as a testing replacement and unlike PGS-M, they can be easily fabricated in the



**Figure 5.10:** Tensile test of  $Ecoflex^{TM}$  00-10 (Smooth-On Inc.),  $Ecoflex^{TM}$  00-30 (Smooth-On Inc.) and PGS-M. The shaded area shows the standard deviation against the mean of the robotic bioreactor.

Sheffield biomedical robotics lab. The IMADA results can also be compared to the first-generation bioreactor results. The results show an average standard deviation of 0.10N/mm for Ecoflex<sup>TM</sup> 00-10 (Smooth-On Inc.) and 0.37N/mm for Ecoflex<sup>TM</sup> 00-30 (Smooth-On Inc.) across the trials(Fig. 5.10). Although the robotic bioreactor results follow the IMADA results to some degree, the relative error for the Ecoflex<sup>TM</sup> 00-10 (Smooth-On Inc.) force measurements was as large as 50%. From conducting the experiments, it was difficult to get the scaffold tensioned in the clamps the same each time before the experiment. This was partly due to how much the scaffold was initially clamped, but it was clear that, due to friction between the hook mechanism and force sensor, the hook would apply a force on the sensor without force being applied on the scaffold. This problem that would occur made the compression force sensor mechanism somewhat unreliable. This was one of the driving experiments that led to the change to the tensile force sensor used in the second-generation robotic bioreactor and also explains why the second-generation bioreactor was not used for this experiment, as it was not developed yet.

The high standard deviation observed in the tensile test results may be attributed to several factors. Ecoflex<sup>TM</sup> samples, although falling within a similar force range, may still exhibit some material variability, contributing to standard deviation. Variations in the preparation of the silicone samples, such as slight differences in dimensions or inconsistencies in material composition, can contribute to variability in the test results. Differences in the testing conditions between the IMADA and the first-generation bioreactor, including loading rates, environmental conditions, and calibration, can introduce variability. Precision and accuracy of the testing instruments (IMADA and first-generation bioreactor) can also contribute to variability. The noted difficulty in consistently tensioning the scaffold in the clamps of the first-generation bioreactor, including issues with friction and hook mechanisms, could introduce variability in force measurements. Challenges in clamping the scaffold consistently in the first-generation bioreactor, as mentioned, could lead to variations in force application and measurement. Operator-dependent factors, such as the manual handling of samples and setting up the experiments, can contribute to variability.

To address this issue in future trials, it may be beneficial to conduct additional trials to increase the sample size and improve statistical reliability. Also, investigate and address specific sources of variability, such as refining the experimental setup, improving clamping procedures, and ensuring consistent sample preparation. Furthermore, analysing and reporting any observed trends or patterns in the data would enable insights into the variability.

The transition to a tensile force sensor in the second-generation bioreactor, as mentioned, suggests that efforts were made to address some of the challenges observed in the first-generation setup.

### 5.3.2 Comparison of two force sensor performance

In this experiment, the linear actuator was used to displace the phantom scaffold for both sensor types. By reducing the number of changed variables in this experiment, a direct comparison of the force sensors can be made. For the first and second-generation force sensor, a ramp signal was implemented in 1mm increments, with a 5-second delay between each step. The phantom scaffolds (Mold Star<sup>TM</sup> 15 SLOW (Smooth-On Inc.) and Ecoflex<sup>TM</sup> 00-50 (Smooth-On Inc.)) were set up three times and for each time the scaffold was set up, the ramp signals (1mm to 5mm to 1mm) were repeated six times. The values at the static positions were averaged over the eighteen trials and then the average and standard deviation were computed and plotted in Fig. 5.11. As the IMADA tensile machine could not implement a pre-programmed displacement routine, the displacement was controlled using the buttons on the IMADA test stand.

In this experiment, the IMADA results are considered the theoretical 'true' measurements which the bioreactor results are compared against. As the silicone samples are fabricated in the lab, fabrication processes, such as curing time and temperature, can impact the material properties. Tab. 5.3 shows the average relative errors across the five displacements,



**Figure 5.11:** Comparison of the two force sensor options, against the IMADA tensile testing machine for an  $Ecoflex^{TM}$  00-30 (Smooth-On Inc.),  $Ecoflex^{TM}$  00-50 (Smooth-On Inc.) and a Mold Star<sup>TM</sup> 15 SLOW (Smooth-On Inc.) phantom scaffold.

**Table 5.3:** Relative error of the first and second generation force sensorsused against IMADA results.

	Average Relative Error (%)			
	$Ecoflex^{TM}$ 00-10	$Ecoflex^{TM}$ 00-50	Mold Star <sup>TM</sup> 15 SLOW	
			SLOW	
First Gen	15	45	32	
(Compression)				
Second Gen	10	21	20	
(Tensile)				

Across the three materials, used the average relative error is lower for the secondgeneration tensile force sensor. This could be attributed to the fact that the secondgeneration tensile force sensor removes the mechanical noise present in the hook mechanism in the first-generation force transduction mechanism and it does not need such a mechanism. Although the error is lower in the second-generation tensile force sensor, measurement errors do still exist. This could be due to multiple contributing factors. The first is that the force sensor range, as described in the datasheet, is 0-5N. In this experiment, a range of 0 - 1.3N of the tensile force sensor is used, so only around 26% of the force range is utilised. Small forces are difficult to measure because they produce small and subtle effects that can easily be overwhelmed by noise and other sources of interference. The sensors themselves are subject to various sources of noise, such as thermal fluctuations and electrical interference, which can obscure the small signals produced by small forces. Furthermore, the accuracy and sensitivity of the instrument used to measure the force can also limit the precision of the measurement. Sensors with higher sensitivities are likely to detect small forces but they are also susceptible to noise and they cost more. The first-generation compression sensor has a sensitivity of 7.2mV and the second-generation tensile sensor has a sensitivity of 2mV. Even with a lower sensitivity the tensile sensor could detect the forces more accurately than the compression sensor due to the high noise in the hook mechanism. However, the cost of the second-generation tensile force sensor is at least four times the price of the first-generation compression force sensor.

The force comparison experiments highlight that regardless of the sensor used, the standard deviation of the force measurements remained relatively high. Measuring a force downstream of where it is acting can lead to inaccurate or misleading results. If the force has been attenuated or dissipated by the time it reaches the measurement point, the measured force may be lower than the actual force that was applied. There could also be a time delay between when the force was applied and when it is measured downstream, the measured force may not accurately represent the actual force that was applied. Other forces or sources of interference, such as the friction forces to move the clamps or the damping effect of the liquid in the chamber, may affect the measured force downstream of where it is acting. These interference forces may add to or subtract from the actual force that was applied, leading to inaccurate measurements.

To improve upon this high standard deviation, several considerations and potential solutions can be explored. By placing the force sensor or measurement point as close as possible to the location where the force is applied. This minimises the potential for force attenuation or dissipation before reaching the measurement point. Implementing real-time force monitoring systems would reduce the time delay between force application and measurement. This ensures that the measured force accurately represents the actual force being applied at any given moment. Another idea would be to identify and minimise sources of interference forces, such as friction forces during clamp movement or damping effects of the liquid in the chamber. Calibration and optimisation of equipment can help mitigate these interference forces. Another option would be to enhance the clamping mechanism to ensure consistent and reproducible tensioning of the scaffold in the experimental setup. This reduces variability introduced during the clamping process. By regularly calibrating and validating force sensors to ensure accurate and reliable measurements. Calibration should be performed under conditions that simulate the actual experimental setup. In addition, standardising the experimental protocols, including sample preparation, loading conditions, and sensor placement, would minimise variability between trials. By addressing these considerations, it is possible to enhance the accuracy and reliability of force measurements in biomechanical experiments, ultimately reducing the observed high standard deviation.

# 5.4 Chapter Summary

In this chapter, three main performance areas are explored and the two prototypes are compared. Firstly, the displacement accuracy of the first and second prototypes was compared for a three-minute experiment and then a three-day experiment. Displacement accuracy refers to the precision with which the bioreactor can measure the amount of deformation or elongation of the tissue being tested. Accurate displacement measurement is essential for the reliable characterization of the mechanical properties of biological tissues, the comparison of results obtained from different tests, and the optimization of tissue engineering. Therefore, displacement accuracy is a critical factor to consider when designing, using, and interpreting data from tensile bioreactors. The results showed that the DC motor from the first prototype showed higher variance than the linear actuator from the second prototype but similar means across different conditions. The displacement accuracy was then compared over a three-day period, where the results showed that the duration of experiments did not alter the results significantly. Although the displacement measurements are relatively accurate, it is difficult to say where they are accurate enough for low-stiffness tissue construct where the errors are larger for small variations.

Secondly, the ability of the chamber to be isolated from the surrounding environment is tested for the first and second prototypes. Tissue engineering bioreactor chambers need to be isolated from the surrounding environment to provide a controlled environment for the growth and development of engineered tissues. The chamber's ability to hold water over a twenty-four-hour period is tested and discussed. The results show how the second prototype membrane design can hold water more effectively across three trials than the first prototype. The deconstructed parts of the second prototype membrane design is tested in extreme conditions and no leaks were recorded. Due to the small variation shown in the experiment, before any in vitro experiment, the chamber membrane should be tested to detect any leaks which could disrupt an in vitro experiment.

Finally, the force measurement accuracy is tested and compared. Force measurement accuracy is important for tissue engineering bioreactors because it is essential for ensuring the reliability and validity of the mechanical testing of biological tissues. Inaccurate force measurements can lead to errors in tissue characterization, making it difficult to draw valid conclusions from the data. Both force sensors showed variation in force measurements which can be explained by the force sensor placement in the bioreactor. The further away the sensor is from the source, the lower the sensor accuracy as there is more likely to be noise in the system. However, moving the sensor inside the bioreactor chamber introduces different challenges, one of which is how to sterilise it if it resides inside the chamber.

# Chapter 6

# Stiffness-Based Control Development

As discussed in the literature review, as cells proliferate and produce ECM, the overall global stiffness of the construct changes. These mechanical changes can be used to track the development of the cell-seeded scaffold without using more sensors which can complicate the design and introduce more factors that can fail. For this reason, the robotic bioreactor needs to be able to use the readings from the force sensor and the motor to calculate the stiffness of the cell-seeded scaffold. In this chapter, only the second-generation bioreactor is used and so any reference to the robotic bioreactor in this chapter is referring to the second-generation robotic bioreactor. Any stimulation regimes applied by the bioreactor in this chapter use position-based control to achieve the desired stimulation.

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**Figure 6.1:** Overall concept for stiffness-based control implemented in the robotic bioreactor.

The overall concept of the robotic bioreactor is summarised in Fig. 6.1.

The stiffness of the cell-seeded scaffold can be calculated as:

$$S_s = \frac{F_s}{E_s} \tag{6.1}$$

where  $S_s$  is the stiffness of the scaffold,  $F_s$  is the force administered to the scaffold and  $E_s$  is the elongation of the scaffold, as transduced by the sensors in the system.



**Figure 6.2:** Block diagram representing the stiffness-based position control implemented into the bioreactor.

# 6.1 Controllable Stiffness Scaffold Phantom

To determine how to best calculate the stiffness during the scaffold stimulation regime, a testing rig was developed utilising a 12V linear solenoid in replace of the chamber, clamps and scaffold.

The linear solenoid is an electromagnetic device that converts electrical energy into a magnetic field that is able to induce a linear mechanical force. The solenoid has a wire coil inside and when a voltage passes through, a magnetic field is produced. The iron core plunger is free to move in and out of the linear solenoid. When a voltage is applied, a magnetic field is generated and the plunger is attracted towards the centre of the coil by the magnetic flux set up within the coil's body, The force and speed of the plunger are dependent on the strength of the magnetic field. The duty cycle is the ratio of time a load or circuit is on compared to the time the load or circuit is off and is used to prevent overheating. It can also be used to directly control the strength of the magnetic field and therefore, the force needed to pull the plunger from the solenoid.

## 6.1.1 Characterisation of the Linear Solenoid

To use the linear solenoid as a controllable stiffness scaffold phantom, it must first be characterised to know the relationship between the duty cycle implemented and the force output of the linear solenoid.

Duty cycle refers to the percentage of time a device or system is in an active or "on" state compared to the total time it is operational. It is expressed as a ratio of on-time to total time, usually as a percentage. It can be calculated using this equation;  $DutyCycle = (ONTime/(ONtime + OFFtime)) \times 100\%$ 

#### Static Linear Solenoid Characterisation

The initial characterisations are performed using the linear solenoid, Arduino Nano and the IMADA tensile testing machine and the experimental setup is shown in Fig. 6.3.

In Fig. 6.4, a static characterisation was conducted where The solenoid plunger was positioned with 45mm inside the solenoid and the duty cycle was increased in increments of ten from 10% to 60% using an Arduino Nano.



**Figure 6.3:** Experimental set-up to characterise the input duty cycle and force output relationship of the linear solenoid.



Figure 6.4: Relationship between the duty cycle input and the measured force when the solenoid plunger is positioned 45mm inside the linear solenoid. IMADA force measurements (orange line) and bioreactor (blue line) force measurements are also compared in this plot.

Fig. 6.4 shows that as the duty cycle increases, the force needed to maintain the plunger at its static position increases at a small gradient at first and then at a greater gradient with higher duty cycle inputs. For this experiment, the bioreactor force measurements closely follow the IMADA force measurements. The plot also shows how magnetic hysteresis affects the force measurements; when the plunger is static, the dashed lines, representing the increasing duty cycles, fall above the solid line, representing the decreasing duty cycles.

This experiment lays the foundation for understanding the relationship between duty cycle input and force output. However, during the robotic bioreactor's stimulation regime, the plunger will be moved into and out of the linear solenoid by the linear actuator of the robotic bioreactor. The force output of a linear solenoid is not only dependent on the duty cycle but also on the position of the plunger within the solenoid's coil. As the plunger moves out of the solenoid coil, the magnetic flux density experienced by the plunger decreases, leading to a decrease in the force output of the solenoid. The force output of a linear solenoid is determined by the magnetic field strength, which is proportional to the current flowing through the solenoid coil. The magnetic field strength is also dependent on the length of the coil and the number of turns in the coil. When the plunger is fully inside the solenoid coil, the magnetic flux density experienced by the plunger is at its maximum, resulting in maximum force output. As the plunger moves out of the solenoid coil, the magnetic flux density experienced by the plunger decreases due to the increased distance between the plunger and the coil. This decrease in magnetic flux density leads to a decrease in the force output of the solenoid. Additionally, the force output of the solenoid decreases as the plunger moves out because the length of the coil effectively decreases, which reduces the magnetic field strength.

Therefore, further investigation needs to be explored to characterise the dynamic relationship between the distance of the plunger out of the linear solenoid, the duty cycle and force output.

#### Dynamic Linear Solenoid Characterisation

This experiment aims to characterise the relationship between the distance of the plunger out of the linear solenoid, the duty cycle and force output. The experimental setup is the same as the setup for the static characterisation, shown in Fig. 6.3. The linear solenoid plunger was set to the zero point and then moved into the solenoid at a constant rate of 50mm/s using the IMADA tensile testing machine. This movement of the plunger moving into the linear solenoid will be referred to as the in-stroke. Once the plunger cannot move inside the linear solenoid anymore, is reaches its endpoint, it is pulled back out of the linear solenoid with the IMADA tensile testing machine. This movement of the plunger moving out of the linear solenoid will be referred to as the set of the solenoid.

3.5 Duty Cycle <u></u>−10% 3 20% 30% 2.5 40% 50% 2 Force (N) 60% 1.5 1 0.5 0 -0.5 └ 30 32 34 36 38 40 42 44 46 48 Displacement (mm)

out-stroke.

**Figure 6.5:** Force and displacement relationship for the linear solenoid changing with duty cycle tested on the IMADA tensile testing machine.

In this experiment, 0mm refers to the plunger being entirely out of the linear solenoid and 48mm refers to the plunger being completely inside the linear solenoid. The force required to move the plunger is recorded and averaged at 1mm intervals across the instroke and out-stroke and plotted in Fig. 6.5. As the plunger moves inside the linear solenoid from the zero position (outside the linear solenoid), there is little change in force until around 30mm stroke is reached as shown in Fig. 6.6. Therefore the force measurement will be plotted from 30mm to 48mm.

The error bars shown are the standard deviation of the data around the mean at the 1mm intervals. Fig. 6.5 shows hows with increasing duty cycle, the force required to move the plunger in and out of the solenoid also increases. A larger standard deviation at larger displacement exists due to the hysteresis effect of magnets [221]. shown in Fig. 6.6 for 40% and 60% duty cycle. Magnetic hysteresis in linear solenoids occurs due to the presence of a material with a magnetic core. When a solenoid is energized, it creates a magnetic field that passes through the core material, causing it to become magnetized. Hysteresis is a phenomenon where the magnetic properties of a material depend not only on the current that created the magnetic field but also on the history of that magnetic field. In the case of a solenoid, this means that the force it can generate on an object may depend on its previous state of magnetization. Therefore the linear solenoid must be characterised separately for its in-stroke and out-stroke.



**Figure 6.6:** Magnetic hysteresis shown for 60% and 40% duty cycle for pulling the plunger out of the linear solenoid (solid line) and pushing the solenoid back into the



**Figure 6.7:** Force and displacement relationship for the linear solenoid out-stoke and in-stroke for a given duty cycle, measured by the IMADA tensile testing machine.



**Figure 6.8:** Rearranged Fig. 6.5 to give duty cycle against the force for a given displacement.

The data obtained from this experiment (Fig. 6.5) was separated into out-stroke and in-stroke. The force and displacement relationship for a given duty cycle for the linear solenoid's out-stroke and in-stroke are plotted separately in Fig. 6.7. The error bars represent the standard deviation around the mean. As the duty cycle increases, the force required to displace the linear solenoid plunger increases as before. The out-stroke uses higher forces to displace the plunger compared to the in-stroke. For example for the 60% duty cycle, the force needed to displace the plunger 45mm in the out-stroke is around 2.4N but only 1.9N in the in-stroke. This is due to the magnetic hysteresis described earlier. The effect of magnetic hysteresis increases with the duty cycle and this is also shown in Fig. 6.7, where for 30% duty cycle, the in force needed to displace the plunger 45mm in the out-stroke is around 0.5N and only 0.4N for in the in-stroke. A difference of only 0.1N for 30% duty cycle compared to 0.5N for 60% duty cycle.

The standard deviations in Fig. 6.7 are small and so only the average at each 1mm displacement are used. The data is replotted in produce 6.8. The data is linearised between increments of 10% duty cycle to capture the general trend of the curve but also to simplify the characterisation.

## 6.1.2 Controllable Stiffness Scaffold Phantom Development



**Figure 6.9:** Second-generation robotic bioreactor attached to the linear solenoid.



**Figure 6.10:** *Electrical schematic of the linear solenoid experimental set-up.* 

For this following section, the linear solenoid replaces the material that is stimulated by the bioreactor, as shown in Fig. 6.9, by using viscoelastic models (2.4) to predict the force produced by the viscoelastic material with respect to time for a given displacement. The base and parts that connect the force sensor to the linear solenoid were 3D printed using a fused deposition modelling printer with PLA. A simple electronic schematic of the controllable stiffness scaffold phantom is shown in Fig. 6.10.

The linear solenoid output force is generated from the viscoelastic models. The model uses the real-time displacement of the bioreactor's linear actuator to predict the force that the modelled viscoelastic material would produce with respect to time for a given displacement. This is a useful tool to assess the bioreactor's capability with a highly controllable soft tissue simulator. As in vitro assessments can be unpredictable, using a highly controllable phantom material in the form of a linear solenoid allows a wide variety of material types and developing stiffness patterns to be tested with the robotic bioreactor programme and identify where the limitations of the device are. The viscoelastic model used in these experiments is the voigt model as it has a closed-form solution, which means that it can be solved analytically without the need for complex numerical methods. This makes it a relatively simple and straightforward model to work with, particularly in comparison to more complex models that may require numerical integration or other advanced techniques.



**Figure 6.11:** Block diagram representing how the linear solenoid is used to control the stiffness measured by the bioreactor.

Fig. 6.11 shows how the system works in a block diagram. The control system previously presented in the report for the bioreactor is implemented. A target strain, s, is inputted by the user which corresponds to a target displacement,  $x_t$ , The error between the target displacement and measured displacement,  $x_m$  is computed and fed into a Proportional controller. The bioreactor moves to minimise the error between  $x_t$ and  $x_m$ . The user also inputs a stiffness, k and a damping ratio,  $\beta$  into the system. These values are inputted into a viscoelastic model (e.g. the voigt model) to simulate the behaviour of soft tissue. These values are chosen specifically to represent specific soft tissues. The displacement of the soft tissue,  $x_m$  as measured by the bioreactor, and the differential of  $x_m$  with respect to time are also inputted into the viscoelastic model. This model produces a target force,  $F_t$ , which along with the displacement of the soft tissue,  $x_m$ , is converted into a duty cycle, d, using a 2D look-up table generated from the data plotted in Fig. 6.8.

This duty cycle is inputted into the linear solenoid and the linear solenoid produces a force that attracts the iron plunger into the linear solenoid which is measured by the bioreactor,  $F_s$ . The bioreactor has both the measured displacement,  $x_m$  and the measured force,  $F_s$  so therefore an estimated stiffness,  $k_m$ , can be computed. This should be equal to the inputted variable k at a given time  $(k = k_m)$ .

#### 6.1.3 Stiffness Computation Method Comparison

There is a huge range of different methods that could be used to measure the stiffness of the tissue construct and the system presented in Fig. 6.11 presents a way to compare various different computations of stiffness. In this section, three methods of stiffness computations are trialled and compared. The equation to calculate stiffness is  $k_m = \frac{F_m}{x_m}$ where  $F_m$  is the measured Force (N) and  $x_m$  is the measured displacement (mm), It is hypothesised here that depending on where and how the stiffness measurement is taken and calculated will affect the accuracy of the stiffness measurement taken. The three methods were chosen as they all used the same sinusoidal wave input but just utilised different parts of the wave for stiffness computation. This reduces the impact of measuring the stiffness in an in vitro experiment where stimulation regimes must be strictly followed to have a scientific contribution to the field and allow comparison with other studies. The hypothesis is that using different parts of the waveform to compute the stiffness will affect the stiffness accuracy and therefore it is important to compare the stiffness parts of the waveform.

The first method of stiffness computation is where the stiffness is calculated over the entire sine wave and averaged (Fig. 6.12a). The second method of stiffness computation is using half of the sine wave, from the trough to the peak, to compute stiffness using the same stiffness formula (Fig. 6.12b). The third method of computation uses the stiffness on the part of the sine wave that has a constant gradient (Fig. 6.12c). These three options were selected as they can be implemented during in vitro experimentation of sinusoidal stimulation regimes without any change to the stimulation regime.



**Figure 6.12:** Stiffness computation locations for a) the entire sine wave, b) the trough-to-peak of a sine wave and c) the stable positive gradient of a sine wave. The x-axis, t, is time (seconds) and the y-axis,  $x_m$ , is displacement (mm).

#### **Constant Stiffness Experiment**

Six different stiffness were inputted into the voigt model  $(k_m)$ ; 50N/mm, 100N/mm, 150N/mm, 200N/mm, 250N/mm and 300N/mm. A 0.1Hz sinusoidal stimulation regime, with a maximum displacement of 5mm was executed for 100 seconds and repeated six times. For the same datasets generated for this experiment, three stiffness computation methods were tested. For each sinusoidal wave, the mean stiffness was computed a) across the entire waveform, b) from the trough to the peak of the wave and c) on the stable positive gradient of the wave. The means for each method were then combined into their own respective dataset. The relative error of the resulting stiffness was calculated and plotted as boxplots in Fig. 6.13.



Figure 6.13: Comparing the relative error of three stiffness computation methods for six different constant stiffness inputs  $(50N/mm \ to \ 300N/mm)$ .

A one-way ANOVA was conducted and showed that over all the six stiffness inputs, using the entire waveform to compute the stiffness gave significantly high relative errors compared to the trough-to-peak method or the stable positive gradient method. There was, however, no significant difference between using the trough-to-peak method and the stable positive gradient method. The relative errors were then computed for the stiffness and summarised in Tab. 6.1. The lower stiffness input gave higher relative errors compared to the higher stiffness inputs. This is expected, as earlier discussed earlier, smaller forces are more difficult to measure so they have generally higher errors associated with measuring them.

Stiffness	Average Relative Error (%)					
Computation	k=50	k=100	k=150	k=200	k=250	k=300
Entire waveform	342.5	124.1	66.4	136.6	75.7	47.4
Trough to Peak	35.6	12.4	13.0	11.8	23.7	3.7
Stable Gradient	37.4	9.4	14.6	12.5	24.7	1.8

**Table 6.1:** Summary of the average relative error for each stiffness input, k(N/mm), and for the three stiffness computational methods.

Following these results, the method of using the entire waveform to compute the stiffness will no longer be considered and omitted in any further stiffness computation comparisons. When using a sinusoidal displacement waveform, the system's response may not be perfectly sinusoidal due to non-linearities. This lead to difficulties in accurately determining the stiffness based on the measured displacement.

#### **Changing Stiffness Experiment**

In this experiment, the input stiffness changes over time. The input stiffness follows a quadratic trajectory starting at 300N/mm, decreasing down to 0N/mm before increasing stiffness back up to 300N/mm over approximately 13 minutes. This experiment is set up to test how the bioreactor measures a changing stiffness over time whilst applying sinusoidal stimulation. A 0.1Hz sinusoidal waveform was used as the stimulation regime with a maximum displacement, at the sinusoidal peak, of 5mm.



Figure 6.14: Top plot shows the force vs time where the blue line shows the solenoid input force and the red line shows the bioreactor measured force. The bottom plot shows the bioreactor displacement measurements as transduced by the linear actuator's potentiometer (green line).

Fig. 6.14 shows a time frame, from around 100 seconds to 650 seconds, of the experiment. The plot shows the solenoid input force, the bioreactor-measured force (top plot) and the bioreactor-measured force (bottom plot). The solenoid input force is generated by the voigt model where k is changing with time and a constant damping ratio, 20Ns/m. The model takes the displacement of the linear actuator of the bioreactor to predict the force that the modelled viscoelastic material would produce with respect to time for a given displacement. This is compared to the force measured by the bioreactor. The force measured by the bioreactor follows the solenoid input forces well. At around 240 seconds, the force drops considerably from about 0.4N to 0.2N and at around 585 seconds the force increases considerably from around 0.2N to 0.4N. As this step is fairly symmetrical across the quadratic trajectory and it is a similar force change, it is likely a result of the linear solenoid characterisation, shown in Fig. 6.8, where the linear regression changes gradient. When the solenoid input force is equal to zero, there is still a force being measured by the bioreactor, that follows the same sinusoidal trajectory as the sinusoidal trajectory of the displacement measurements. Although it is not possible to measure a force if no force is being applied, gravity and friction can impact the force measurements. Gravity is a fundamental force that is always present and exerts a force on all objects with mass. Weight is a measure of the gravitational force acting on an object, and it is proportional to the mass of the

object. When an external force is applied to an object, the force must be greater than the weight of the object in order to overcome the force of gravity and move the object. Given that the linear

Friction is a force that resists motion between two surfaces in contact. So, if an object is placed on a surface with friction, such as a table or a floor, and then pushed lightly, it may appear that a force is being measured even though no external force is being applied. In reality, the force of friction is acting between the object and the surface, and this is what is being measured. In summary, while it may appear that a force is being measured even though no external force is being applied, there is always some underlying force or forces that are causing the measurement to occur.



**Figure 6.15:** The input stiffness plotted by the purple line follows a quadratic trajectory in the range of 0-300N/m. The measured stiffness is plotted with red circles using a) the trough to the peak area of the sinusoidal wave and b) the stable positive gradient area

# 6.2 Soft Phantom Scaffold

To test the robotic bioreactor with a less controllable substrate, a stiffness-changing soft phantom scaffold was fabricated and tested.



**Figure 6.16:** Image of the a) PLA mould for the soft phantom scaffold, b) the cured  $Ecoflex^{TM}$  00-50 (Smooth-On Inc.) mould with a shelled area and c)  $Ecoflex^{TM}$  00-50 (Smooth-On Inc.) filled shell.

To achieve this, a PLA mould was printed using the PRUSA 3D printer, as shown in Fig. 6.16a. For both the Ecoflex<sup>TM</sup> 00-30 and Ecoflex<sup>TM</sup> 00-50 (Smooth-On Inc.) the same protocol was followed. The two parts of Ecoflex<sup>TM</sup> were mixed in equal parts and degassed for 3 minutes. The silicone was poured into the PLA mould and left overnight at room temperature to cure to create an Ecoflex<sup>TM</sup> mould. This mould has a shelled body so that uncured Ecoflex<sup>TM</sup> can be poured in during an experiment. This uncured Ecoflex<sup>TM</sup> will cure during the experiment and increase the stiffness of the overall scaffold as the Ecoflex<sup>TM</sup> changed from a viscous liquid to a solid elastomer. The robotic bioreactor will be able to measure the change in stiffness as the Ecoflex<sup>TM</sup> cures. The Ecoflex<sup>TM</sup> mould is then clamped into the robotic bioreactor chamber using the bioreactor clamps and screwed together to ensure the Ecoflex<sup>TM</sup> mould is gripped effectively throughout the experiment. The Ecoflex<sup>TM</sup> mould was then tensioned using the bioreactor buttons under a measurable force applied.

The robotic bioreactor executed an intermittent sinusoidal stimulation regime. Intermittent was chosen to allow some time for the uncured  $\text{Ecoflex}^{\text{TM}}$  00-50 (Smooth-On Inc.) to remain static and disperse evenly across the entire shelled area of the  $\text{Ecoflex}^{\text{TM}}$ mould. The  $\text{Ecoflex}^{\text{TM}}$  soft phantom scaffold was stimulated for 10 seconds at 1Hz with a maximum 2mm displacement and then left static for 30 seconds. One sample of  $\text{Ecoflex}^{\text{TM}}$  00-30 (Smooth-On Inc.) and one sample of  $\text{Ecoflex}^{\text{TM}}$  00-50 (Smooth-On Inc.) were trialled.

The experiment was started and after five minutes of stimulation, pre-mixed Ecoflex<sup>TM</sup> 00-50 (Smooth-On Inc.) was poured into the shelled vacant area of the Ecoflex<sup>TM</sup> 00-50 (Smooth-On Inc.) mould and the measurements were plotted in Fig. 6.17. The displacement follows the sinusoidal stimulation trajectory and peaks at maximum displacement (2mm), the force also peaks. These peaks were isolated in MATLAB, averaged with a rolling average and plotted in Fig. 6.17. The stiffness measurements were conducted approximately every 15 minutes in the stable positive gradient of the sinu-



**Figure 6.17:** The force measurements (top plot) and the stiffness measurements (bottom plot) measured by the robotic bioreactor for a)  $Ecoflex^{TM}$  00-30 (Smooth-On Inc.) is curing within an  $Ecoflex^{TM}$  00-30 (Smooth-On Inc.) mould and b)  $Ecoflex^{TM}$  00-50 (Smooth-On Inc.) is curing within an  $Ecoflex^{TM}$  00-50 (Smooth-On Inc.) mold.

soidal wave, as described earlier in the chapter, and then averaged. Three consecutive wave sines were used to compute the stiffness and averaged.

For Ecoflex<sup>TM</sup> 00-30 (Smooth-On Inc.), the force peaks in Fig. 6.17 start at around 1N and then started to increase at around the two-hour mark. After two hours, the force peaks settled at around 1.5N. The stiffness measurements follow a similar trajectory starting at around 0.5N/mm and increasing to 0.8N/mm.

For Ecoflex<sup>TM</sup> 00-50 (Smooth-On Inc.), the force peaks in Fig. 6.17b start at around 1N and then at around 45 minutes into the experiment, the force rises to around 3N in approximately thirty minutes following a cubic trajectory. The stiffness was computed every 15 minutes following the same trajectory starting at around 0.4N/mm, increasing to approximately 1.4N/mm over the same thirty minutes. This behaviour is expected as the maximum displacement (2mm) remains the same throughout the experiment, so an increase in force would directly increase the stiffness of the soft construct.

From Fig. 6.17, it is clear that the  $\text{Ecoflex}^{\text{TM}}$  00-30 (Smooth-On Inc.) phantom scaffold cure time was around four times longer than that of the  $\text{Ecoflex}^{\text{TM}}$  00-50 (Smooth-On Inc.). On the datasheet, for  $\text{Ecoflex}^{\text{TM}}$  00-30 (Smooth-On Inc.), a cure

time of 4 hours is given compared to the datasheet for  $\text{Ecoflex}^{\text{TM}}$  00-50 (Smooth-On Inc.) where a cure time of 3 hours is given. Factors such as room temperature and volume may have affected the cure time but the results are consistent in that  $\text{Ecoflex}^{\text{TM}}$  00-50 (Smooth-On Inc.) took less time to cure.

For the Ecoflex<sup>TM</sup> 00-30 (Smooth-On Inc.) phantom scaffold taking stiffness measurements every 15 minutes captured the stiffness development over time. However, for the Ecoflex<sup>TM</sup> 00-50 (Smooth-On Inc.) phantom scaffold, during the curing period, only one stiffness measurement captured the stiffness transitional phase. In this case, stiffness computations taken in shorter intervals than 15 minutes would have better captured the transitional stiffness phase of Ecoflex<sup>TM</sup> 00-50 (Smooth-On Inc.) but the same shorter intervals would have been excessive forEcoflex<sup>TM</sup> 00-30 (Smooth-On Inc.). This experiment does highlight the importance of sampling the stiffness at an appropriate rate. Sampling too slow could lead to missed stiffness changes and result in delayed control responses. Sampling too often increases computational requirements. Higher sampling rates mean that more data needs to be processed in a shorter period. This can place a significant strain on the computational resources of the system, potentially leading to slower response times. The latter is unlikely but should be considered. In this case but as the in vitro experiments can last as long as two weeks, factors such as data storage on the Raspberry Pi, are more likely to be an issue.

Using every wave to compute the stiffness for in vitro experiments would be unnecessary as the stiffness change in a cell-seeded scaffold would be very slow. However, making sure that sufficient data is captured to allow debugging and analysis is also very important and so a tradeoff must be made. The continuous change in stiffness during the in vitro experiments, as observed in Fig. 6.17, presents a challenge in precisely determining the true stiffness values at any given moment. The evolving nature of the cell-seeded scaffold makes it difficult to pinpoint exact stiffness values, and the dynamic changes in mechanical properties may not be fully captured by intermittent measurements. Therefore, it is acknowledged that the real-time stiffness of the soft phantom scaffold is continuously changing, and the recorded values represent snapshots of this dynamic process. The tradeoff between capturing sufficient data for analysis and the practical limitations imposed by computational resources is crucial in striking a balance for meaningful experimentation and control responses.

# 6.3 Chapter Summary

The stiffness of a cell-seeded scaffold can be difficult to measure. The scaffold is typically made up of a complex three-dimensional structure, which can be composed of various materials with different mechanical properties. This can make it difficult to obtain an accurate measure of the overall stiffness of the scaffold. The presence of cells within the scaffold can also complicate stiffness measurements. Cells can adhere to the scaffold surface, secrete extracellular matrix proteins, and remodel the scaffold over time, leading to changes in its mechanical properties. The measurement techniques used to assess stiffness can also impact the accuracy of the results. Some techniques require the application of external forces or strains, which can alter the scaffold's structure and mechanical properties. Other techniques, such as atomic force microscopy, may not be suitable for measuring the stiffness of three-dimensional structures. There is currently no standardized method for measuring the stiffness of a cell-seeded scaffold and therefore researchers need to carefully consider these factors when selecting and interpreting stiffness measurement techniques to obtain meaningful results.

The 12V RS linear solenoid was used to develop the stiffness-based control capabilities with a controllable unit before delving into more experimental setups and the complexities introduced by the cell-seeded scaffold.

The stiffness computation comparisons show the importance of how the stiffness is computed and which section of data is used to compute the stiffness. Further work using other signals to compute the stiffness of the material should be conducted and compared to the results presented in this chapter. The results from the soft phantom scaffold highlight the importance of stiffness measurement frequencies and should be adapted depending on the experiment. The decision on sample frequency for stiffness measurements depends on the specific characteristics of the material or system being studied, as well as the goals of the experiment. If the stiffness changes in the cell-seeded scaffold are gradual and occur over an extended period, a lower sample frequency might be sufficient to capture the overall trend. On the other hand, if stiffness transitions are rapid or involve subtle variations, a higher sample frequency may be more appropriate. Another factor in determining the frequency of stiffness measurement is to consider the goals of the experiment. If the primary objective is to capture the general trend in stiffness changes, a lower sample frequency may be acceptable. However, if the goal is to precisely capture transient or rapid changes, a higher sample frequency may be necessary. Another consideration would be to assess the computational resources available for processing and storing data. Higher sample frequencies generate more data, requiring increased processing power and storage capacity. Ensure that the chosen frequency is manageable within the constraints of the experimental setup. Furthermore, the overall duration of the in vitro experiments would need to be considered. If experiments last for an extended period, data storage capacity and computational efficiency become more critical. Balancing the need for detailed information with practical considerations is essential for long-term experiments. An evaluation of the response time of the robotic bioreactor or control system needs to be developed for a given experiment. If rapid adjustments based on stiffness changes are required, a higher sample frequency might be necessary to enable timely responses. Pilot experiments with different sample frequencies to assess their impact on the results could also be conducted. This allows for empirical observation of how changes in sample frequency affect the accuracy and

reliability of stiffness measurements.

In summary, there is no one-size-fits-all answer, and the choice of sample frequency should be made based on a careful consideration of the specific characteristics of the material, the experimental goals, available resources, and the dynamics of stiffness changes over time. Adjustments may be necessary during the experimental design phase or based on initial observations to optimise the sampling frequency for meaningful and practical results. There could also be scope to introduce a control feedback loop that considers the rate of change of stiffness and adjusts the frequency of stiffness measurements accordingly.

# Chapter 7

# Development of Robotic Bioreactor In Vitro Protocols

In this chapter, the development towards an in vitro protocol for the bioreactor is presented and discussed. The work in this chapter was completed with the support of Jeerawan Thanarak. Jeerawan conducted the in vitro procedures and cell analysis and I conducted all of the robotic bioreactor set-up and data analysis that are described in this chapter. This section was developed alongside prototype development, as the protocols explored in this section informed design changes in the robotic bioreactor. It will be clearly stated for each experiment which generation of the robotic bioreactor was used (Tab. 7.1) and what informed design changes arose from the experiment (Tab. 7.2).

The goal of the robotic bioreactor ultimately is to mechanically stimulate cells, to increase cell metabolic rate, cell proliferation and protein secretion whilst sensing the global changing mechanical properties of the cell-seeded construct. In vitro testing is used to investigate and analyse cell behaviour in response to mechanical stimulation. To reach this goal, in vitro protocols must be developed that are suitable for the bioreactor and also effective for successful in vitro experimentation.

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#### Arduino and **Raspberry Pi** motor controller Incubator 5% CO2 37°C 0% Humidity 0 С 0 0 Motor Force Bioreactor Sensor Chamber

# 7.1 Robotic Bioreactor In Vitro Set-Up

**Figure 7.1:** Electronic schematic of the robotic bioreactor in vitro experimental set-up where the motor, force sensor and chamber are positioned inside an incubator.

For long-term experiments, it is important that the data is well organised and saved often as it is likely that loss of data will be detrimental to the quality of the scientific contribution of the research. For this reason, automatic data saving via a Raspberry Pi was implemented and trialled so that no data was lost during experiments. Data is saved to the cloud so that analysis of the experiment can be performed away from the experiment. This is even more crucial during COVID-19 when lab access was restricted.

**Table 7.1:** Summary of the key differences between the first and secondgeneration robotic bioreactors. Specifically in terms of the chamber material, the motor used and the force sensor used as described in Ch. 3.

Generation	Chamber Materia	l Motor	Force Sensor
First Gen	ABS	DC Motor & En-	Honeywell
Robotic		coder	FSS005WNGT
Bioreactor			
Second Gen	Dental SG Res	n Linear Actuator	Richmond Industries
Robotic	(Formlabs)		900 Series low capac-
Bioreactor			ity load cell (RI 900)

A simple electronic schematic is shown in Fig. 7.1. The motor and force sensor are

labelled generically and illustrated as blocks as the specific components change from the first-generation robotic bioreactor components to the second-generation robotic bioreactor, as described in Ch. 3, in different in vitro experiments. These components are summarised in Tab. 7.1.

The users of this bioreactor may not feel confident editing the core code to suit their experiment. For this reason, a comprehensive MATLAB GUI was developed to enable the user to edit the initial conditions of the experiment with ease (Fig. A.4).

# 7.2 In Vitro Protocols

For the experiments described in this chapter, the same in vitro protocols were followed.

## 7.2.1 Cell Culture Protocol

The Human Dermal Fibroblasts (HDF) were obtained with informed consent and ethical approval (NHS research ethics committee # 21/NE/0115)) and processed and stored in accordance with the Human Tissue Act 2004. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 x 10<sup>-3</sup> M glutamine, 100 IU ml-1 penicillin, 100 µg ml1 streptomycin and 0.625 µg ml-1 amphotericin B was used to culture the HDF. Using trypsin (0.025%)/EDTA (0.01%) solution, cultured HDF were harvested, and 1 × 105 cells were seeded in each well of a 6-well plate, or on sterile PGS-M scaffolds. 1x10<sup>5</sup> cells were seeded onto the PGS-M scaffolds and left overnight inside an incubator at 37°C, 5% CO2 for the cells to attach to the scaffolds. The cell-seeded scaffolds were then submerged in the culture media. This cell culture protocol was conducted by Jeerawan Thanarak.

### 7.2.2 Sterilisation of Bioreactor Chambers

**IPA** To disinfect using 70% isopropyl alcohol (IPA), the part was submerged in IPA for 30 minutes and then left under a class II biosafety cabinet to evaporate. 70% IPA was used since it is reported to disinfect more thoroughly in comparison with 70% ethanol [222].

Autoclave Sterilisation using an autoclave was achieved by steam sterilization at 110°C at 15psi.

**IPA and UV Lght** For sterilisation using IPA and UV light, under a class II biosafety cabinet, the chamber and clamps were submerged in fresh 70% IPA for 30 minutes for disinfection and then left out under the biosafety cabinet for the IPA to evaporate for 4 hours. Thirty minutes submerged in ABS was deemed appropriate for disinfection, but on reflection may have adversely affected the material properties. Once the IPA has fully evaporated. The chamber clamps were left under an inbuilt UV lamp (UV-C 254nm, 30W) in the biosafety cabinet, for 30 minutes. Using UV light to sterilise is also known as Ultraviolet germicidal irradiation (UVGI) and works by actually attacking the DNA and RNA of microbes and causing cell death [223]. Jeerawan Thanarak conducted these sterilisation procedures.

### 7.2.3 Stimulation Regime

All experiments described in Tab. 7.2 used a 10-second stimulation using a sinusoidal stimulation followed by a 30-second rest regime. The first set was stimulated by the robotic bioreactor, the second was stimulated by the Ebers chamber (as reference) and the last set of two scaffolds was not mechanically stimulated, and left static. All three sets were kept within the same incubator. The bioreactor set-up and implementation of stimulation regimes was conducted myself. The experiments ran in the controlled environment provided by an incubator; body temperature  $(37^{\circ}C)$ , 5% CO<sub>2</sub>, and 21% O<sub>2</sub>.

## 7.2.4 Cell analysis

Two different assays was be used to evaluate the performance of stimulation; resazurin and picrosirius red assay. Resazurin is a redox indicator that is commonly used in cell viability assays, including in tissue engineering protocols evaluating cell proliferation and bacterial growth [224]. Resazurin can also be used to monitor cell viability and proliferation in bio-engineered organ constructs [225]. The cells were stained in 0.2 mg/ml resazurin in the medium for 4 hours on a rocker within an incubator. The aliquot from each well will then be transferred to the 96-well plate before being read with the fluorescent plate reader.

To identify and quantify collagen fibres in samples of tissue, the Picrosirius red assay is commonly used [226]. Phosphate-buffered saline (PBS) was used to wash the scaffolds or cells growing on TCB. Formaldehyde (4%) was then used to fix the cells. Scaffolds or cells growing on tissue culture plastic (TCP) were washed with PBS and fixed with 4% formaldehyde for 20 min, before being washed 3x in PBS. To quantify collagen production, the cells were stained for 12 hours with 0.1% Picrosirius red solution (0.1% Direct Red 80 in saturated picric acid). The stain was then removed with deionized water and then with methanol:0.2 M sodium hydroxide (1:1) for 30 minutes on a rocker. The fluorescent absorbance was then measured, at 490 nm, of the resulting solution. The assays were performed by Jeerawan Thanarak.

## 7.3 Experimental Protocol Exploration

This section outlines an unseeded scaffold experiment, a sterilisation experiment and a material toxicology assessment.

### 7.3.1 Unseeded scaffold experiment

An experiment to test the functionality of the bioreactor in cell culture conditions (inside an incubator) over 24 hours was conducted with the first-generation bioreactor. An unseeded PGS-M scaffold, measuring  $20 \text{mm} \times 10 \text{mm} \times 1 \text{mm}$  was mounted to the bioreactor clamps. Water was added to the chamber to prevent the scaffold from drying out and to simulate the cell media that will be used once cells are applied. The scaffold was left in the chamber of the bioreactor for one day. No sterilisation process was needed as the scaffold was not seeded with cells. During the twenty-four hours, the robotic bioreactor implemented an intermittent cyclic stimulation regime based on a sinusoidal waveform with a frequency of 0.1Hz and 5% scaffold extension (5mm). The 0.5mm displacement falls in line with extensions used in Tab. 2.2 of the original length. The scaffold was stimulated for 10 seconds, followed by a 30-second rest where there is no stimulation applied to the scaffold. Given that the duration of the experiment is 24 hours, scaffold degradation should not affect the results. As there are no cells seeded onto the scaffold in this experiment, cell action should not affect the results. Therefore, it is expected that the force sensor measures a consistent force across the entire experiment.



**Figure 7.2:** Displacement and force reading from the 24-hour unseeded cell experiment in cell culture conditions. 10-second windows are shown (stimulation period) at a) 1 hour, b) 10 hours and c) 20 hours, offset from the start of the experiment to show how the force readings changed throughout the experiment.

Fig. 7.2 presents the displacement and force measurements obtained from the robotic bioreactor over the 24 hours at three times offset from the start. Fig. 7.2a) shows the results at approximately 1 hour into the experiment. The plot shows clearly that the force readings follow the displacement trajectories very well. The no stimulation of the base force reading lies at around 0.8N. This can be explained by the pre-tensioning of the scaffold at the start of the experiment. In Fig. 7.2b), approximately 10 hours into the experiment, the force sensor readings remain high and no longer follow the sinusoidal trajectory. In Fig. 7.2c), approximately 20 hours into the experiment, the force reading follows the sinusoidal trajectory.

The observed divergence in force sensor readings between 10 and 20 hours into the in vitro experiment may be attributed to potential experimental or setup errors. Variability in the initial pre-tensioning of the scaffold, inconsistent clamping mechanisms, or the possibility of calibration drift in the force sensor could introduce inaccuracies in force measurements. Fluctuations in environmental conditions, such as temperature or humidity, may impact the mechanical properties of the scaffold material. Additionally, aging or wear of the scaffold over the course of the experiment could contribute to changes in its mechanical behavior. It is essential to scrutinize the stability of the experimental setup, assess the performance of the robotic bioreactor and control sys-
tem, and conduct control experiments to isolate potential sources of error. Regular checks on instrumentation calibration and thorough monitoring of the experimental conditions are crucial to enhance the reliability and consistency of force measurements in in vitro experiments.

From this experiment, the functionality of using the electronic components within an incubator was confirmed as the components were still functional after 24 hours and the unseeded PGS-M scaffold was still intact. At the end of the experiment, the scaffold seemed to have sagged in comparison to the start. This may indicate that the scaffold was plastically deformed, in which case the maximum extension was too high, the initial tensioning was too high or a glitch caused excessive movement of the clamps. There were no indications of a glitch in the displacement measurements obtained from the bioreactor. This hypothesis, however, does not explain why the force sensor readings were slightly higher at the end of the experiment compared to at the start of the experiment. If the scaffold had sagged, it would be expected that the force reading would have been lower at the end of the experiment. Friction forces existing between the moving parts could explain this, if, after the glitch, the position of the force sensor had changed slightly. However, if this is the case, it raises the question of whether the force sensor can capture any relevant forces from the scaffold and whether the friction forces are too high in relation to the scaffold stiffness.

#### 7.3.2 Sterilisation Experiment

Different methods can be used to sterilise equipment that comes into contact with cells. Effective methods discussed in the literature review include autoclaving. With the ABS material used to fabricate the first-generation chamber and clamps, using the autoclave to sterilise the chamber and clamps, was not deemed suitable due to the material's softening point (105°C) [227]. The dental SG resin, used for the fabrication of the second-generation chamber and clamps, is deemed to be autoclavable [228].

The sterilisation experiment conducted compares the two methods of sterilisation for the second-generation chamber, fabricated from the dental SG resin with the gasketbased design for the chamber membrane. The first method is using the autoclave and the second method uses isopropyl alcohol (IPA) and ultraviolet (UV) light to sterilise the chamber and clamps. For this experiment, one second-generation chamber will be autoclaved and the other submerged in IPA and then sterilised using a UV lamp following the protocol described in Sec. 7.2.2.



Figure 7.3: Images of the two chambers (Dental SG resin (Formlabs)) were sterilised using a) the autoclave and b) IPA and UV light.

Images of the chambers that were sterilised can be seen in Fig. 7.3. As shown in Fig. 7.3a, after autoclaving, the material changed colour to a lighter yellow and became a lot more brittle. The corner which allows the lid to be bolted onto the chamber cracked off. The acrylic washers, used in the gasket-based design for the membrane, and the acrylic lid were warped during autoclaving and therefore, the sealing of the chamber was no longer effective. Therefore, no further experimentation could be conducted with the chamber that had been autoclaved. The sterilisation experiment continued with the chamber that had been sterilised with IPA and UV light and with the static control in the 6-well plate. This experiment was conducted twice with IPA and UV light, labelled 'UV sterilisation 1' and 'UV sterilisation 2' in Fig. 7.5.

A contamination test was then conducted, by Jeerawan Thanarak, with the chamber fabricated from Dental SG resin (Formlabs) from the second-generation robotic bioreactor using IPA and UV light, to confirm whether the robotic bioreactor is sterile during an in vitro experiment and can be in contact with cells without contaminating them. This experiment was repeated twice. In this experiment, the cell culture media used is Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum,  $2 \ge 10^{-3}$  M glutamine, 100 IU ml-1 penicillin, 100 µg ml1 streptomycin and 0.625 µg ml-1 amphotericin B. The unseeded scaffolds and culture media were left inside the bioreactor for 3 days. Cell culture media was also added to a well plate and also left for three days in an incubator as a control.

After three days, a sample of the cell culture media was taken from the chamber and then added to a different well of a six-well plate. The cell culture media from the chamber and control was stained with resazurin following the protocol described in Sec. 7.2.4. The aliquot from each sample was then transferred to the 96-well plate before being read with the fluorescent plate reader. If there was no contamination and no cells added then the metabolic activity should be the same as the control.



**Figure 7.4:** Well plates with cell media collected after three days with added resazurin. 1) Control cell media that will act as a background for the analysis with resazurin (purple colour). 2 & 3) Wells contain cell media samples taken from the UV sterilised chambers 3 days after it was added with resazurin added (pink colour).

Fig. 7.4 shows the well plates and Fig. 7.5 shows the results from the resazurin assay conducted to test for the presence of bacteria. The colouration of the cell media with resazurin is an immediate indicator of metabolic activity as a dark purple indicates no metabolic activity and pink indicates high levels of metabolic activity (Fig, 7.4 which is confirmed by the data obtained from the fluorescent plate reader. The data in Fig. 7.5 was obtained from the fluorescent plate reader and shows that there was some metabolic activity in the media when compared to the control as a background. This indicates the presence of bacteria in both UV sterilisation attempts as there were no cells present so metabolic activity can only be explained by the presence of bacteria. This shows that further work needs to be conducted to determine whether IPA and UV sterilisation can be reliably used for in vitro experiments as they have shown to be effective in other studies. As UV light can only sterilize surfaces that are directly exposed to the light, it may not be effective for equipment with complex shapes or hard-to-reach areas, as reported in [229] such as the bioreactor chamber.



**Figure 7.5:** Results from the resazurin assay following the UV sterilisation of the second-generation robotic bioreactor chamber. Three samples from both UV sterilisation experiments were taken and the standard deviations are shown in the error bars.

### 7.3.3 Material toxicology Assessment

To test whether the materials are inert and non-toxic to cells, a toxicology assessment was conducted for the materials in contact with the cells during an in vitro experiment. The experiment used the second-generation bioreactor, where dental SG resin was the main material of the chamber. Cell culture media was prepared by combining Dulbecco's modified eagle's medium (DMEM) with Fetal Calf Serum, penicillin, fungizone and glutamine. A cell-seeded scaffold and culture media were then added to 1) the robotic bioreactor chamber and 2) a well plate (a positive control). Cell culture media was also added to a blank well (no cells). The media was changed three times over the seven-day experiment. On the last day of the experiment, the cells were stained in 0.2 mg/ml resazurin in the medium for 4 hours on a rocker within an incubator. The aliquot from each well will then be transferred to the 96-well plate before being read with the fluorescent plate reader. Resazurin was used to confirm the cell metabolism [224] compared to the tissue culture plate (TCP) control, shown in Fig. 7.6. This experiment was repeated twice, as shown in Fig. 7.6 and the standard deviation is shown in the error bars. Jeerawan Thanarak conducted this cell assay and produced the plot shown in Fig. 7.6.



Cell metabolic rate as a percentage of 2D control from individual experiment (resazurin).

**Figure 7.6:** Cell metabolic rate as a percentage of 2D control from individual experiment (resazurin). Standard deviations are shown in the error bars. Plot generated by Jeerawan Thanarak.

There was no significant difference between the positive control and the robotic bioreactor chamber so we can confirm that the robotic bioreactor chamber did not adversely affect the cell. However, two experiments are not enough repeats to fully rely on the results so more repeats of this experiment need to be conducted for final versions of the robotic bioreactor to confirm. However, as the dental SG resin has a biocompatibility certificate [216], it supports the hypothesis that the cells were not adversely affected by the robotic bioreactor chamber.

## 7.4 In Vitro Experimentation

Throughout the project, three in vitro experiments were conducted and are summarised in Tab. 7.2 for the sinusoidal stimulation applied, strain, length of the experiment, reason the experiment ended and the main action taken as a consequence of the experiment. As these experiments were primarily used to inform design changes of the bioreactor, repeats of experiments were limited to save resources and time.

In all experiments detailed in Tab. 7.2, the same cell culture and analysis protocols, as described above in Sec. 7.2 were followed.

**Table 7.2:** Overview of the in vitro experiments that were performed. All experiments used HDF seeded on a PGS-M scaffold. The table outlines; the experiment label, the generation of the robotic bioreactor used in the experiment (as summarised in Tab. 7.1), the frequency of the sinusoidal stimulation, the maximum strain applied to the scaffold, the length of the experiment, the reason for ending the experiment and the action taken to improve the device/ protocol for following experiments.

Exp	Generation	Freq	Strain	Length	Reason for End-	Action
Ref	of robotic			of Exp	ing Exp	Taken
	bioreactor					
1	First Gen	1Hz	10%	3 days	Cell media showed	Change
					high pH on day 3	incubator
						used in
						$\exp$
2	First Gen	0.5 Hz	5%	5 days	Cell media leaked	Material
					out of chamber	changed
					floor by day 5	to dental
						resin SG
3	Second Gen	1Hz	5%	10 days	Significant signs of	Test Ster-
					contamination on	ilisation
					day 10	methods

### 7.4.1 Experiment 1

For experiment 1, the first generation bioreactor was used, which is described in Tab. 7.1. One HDF-seeded PGS-M scaffold culture with the cell culture protocol described in Sec. 7.2.1 was added to a well plate to act as a control, one was added to the Ebers chamber and one was added to the robotic bioreactor chamber as shown in Fig. 7.7. Both the scaffolds in the Ebers and bioreactor were stimulated with a 1Hz sinusoidal stimulation waveform with a 10% strain. The scaffold in the well plate was left static.



**Figure 7.7:** Experimental Setup of Experiment 1. Control in yellow, Ebers TC-3 in green and the robotic Bioreactor in pink.

The carbon dioxide  $(CO_2)$  sensor in the incubator was faulty and did not release enough  $CO_2$ .  $CO_2$  is used to maintain a constant pH level in cell media. Equation 7.1 shows how the  $CO_2$  dissolved in water releases H<sup>+</sup> which is known to determine pH.

$$H_2CO_3 \rightleftharpoons HCO_3^- + H^+ \tag{7.1}$$

The cell media has a phenol red pH indicator mixed in, so a colour change indicates a change in pH. In this experiment, the colour of the media changed to a dark red in all cases (static, Ebers and robotic bioreactor) indicating a high pH. To test whether the cells could survive in such conditions, HDF cultured on a 2D petri dish were left in the incubator used in the prior experiment and also in a different incubator and left overnight. They were then examined under a microscope and images taken through the microscope are shown Fig. 7.8. After inspection under a microscope, the HDF that were left overnight in a known working incubator were still attached to the TCP (Fig. 7.8a) and therefore, still alive. This acts as our control and confirms that the cells were viable in a working incubator. The cells that had been left in the incubator used in experiment 1 had detached from the 2D surface indicating cell death (Fig. 7.8b).



Figure 7.8: Image of human dermal Fibroblasts on a 2D petri dish in a) a Thermo Fisher working incubator and b) the Ebers incubator used in experiment 1. Human dermal Fibroblasts from primary dermal tissue were obtained with informed consent (REC 15/YH/0177) and processed and stored following the Human Tissue Act 2004.

### 7.4.2 Experiment 2

For experiment 2, the first generation bioreactor was used, which is described in Tab. 7.1. Three PGS-M scaffolds were seeded with HDF following the procedure described earlier in Sec. 7.2.1. One was added to a static well plate, one was added to the Ebers chamber and then one was added to the robotic bioreactor chamber. In this experiment, due to a fault in the Ebers bioreactor, the scaffold in the Ebers chamber was left static with no stimulation applied. The TCP control in this experiment refers to a well plate that has had media added to the well plate but no scaffold and so acts as a control.

The experiment was stopped on day five when it was noticed that a significant amount of cell media had leaked out of the chamber of the robotic bioreactor. This meant that not only were there not enough nutrients available for the cells but also that the chamber was not entirely sealed from the outside environment. A cell-seeded scaffold was also placed inside the Ebers chamber, which was used as a positive control. By using a commercial bioreactor as the positive control, the results from the robotic bioreactor can be directly compared to an industry-standard tool and therefore validate the performance of the robotic bioreactor. The same type of media was added to the 6-well plate for the same length of time. A resazurin and picrosirius red assay was conducted following the protocol described in Sec. 7.2.4. The results from the resazurin assay and the picrosirius red assay are shown in Fig. 7.9 and Fig. 7.10. For both figures, only the TCP has error bars (standard deviation), where there were three samples, and the others only had a sample of one, so no statistical analysis could be made.

Comparison of cell metabolic rate between stimulated scaffold (robotic bioreactor), static env. scaffold and 2D control



**Figure 7.9:** Comparison of cell metabolic rate between stimulated scaffold (robotic bioreactor), static scaffold (Ebers) and the 2D control in experiment 2. Plot generated by Jeerawan Thanarak.





**Figure 7.10:** Comparison of collagen production between the TCP control (cell media with no seeded scaffold), stimulated seeded PGS-M scaffold (robotic bioreactor), static scaffold (Ebers) and the 2D control experiment 2. Plot generated by Jeerawan Thanarak.

Fig. 7.9 shows that at the end of the experiment, there was little cell activity in the robotic bioreactor chamber. As the chamber was leaking, this resulted in the cells not receiving the appropriate nutrient to survive and at the endpoint analysis, most of the cells had already died. In Fig. 7.9, the higher value in the Ebers could be a result of

statistical variation as the sample size was one. Furthermore, as it can be seen that cells survived in the other conditions, it is confirmed that the cells were viable when first seeded.

Fig. 7.10 shows that although no cells survived at the end of the experiment, during the experiment, cells did produce collagen. It would be expected that the stimulated scaffold would produce more than the static. However, in [230], HDF were cultured statically and dynamically using medium flow to stimulate the cells. Sixteen samples were cultured and four samples were analysed on day 3, another four on day 7, another four on day 14, and the remaining four on day 21. Their collagen production, along with other parameters, was quantified for each set. The collagen quantity only significantly increased at day 14 when the HDF were also shown to have reached confluence. This could indicate that the five-day period in experiment 2 was too short for any increase in collagen to be detectable.

In Fig. 7.9 and Fig. 7.10, the values in Ebers are both highest even though the scaffold was left static and therefore, it would be expected that the result would be similar to that of the static well plate. The higher value in the Ebers could be a result of statistical variation as the sample size was one or more cell media added to the Ebers chamber in comparison to the well plate given the larger area in the chamber.

#### 7.4.3 Experiment 3

For experiment 3, the second generation bioreactor was used, which is described in Tab. 7.1. HDF were cultured using the protocol described in Sec. 7.2.1. As summarised in Tab. 7.2, the experiment was stopped after 10 days due to significant signs of contamination. Before the experiment was stopped, stiffness measurements were taken every two hours, as discussed in Sec. 6.1.3, during an in vitro experiment, plotted against time in Fig. 7.11.



**Figure 7.11:** Stiffness measurements were taken every two hours during an in vitro experiment (experiment 2) and plotted against time for 260 hours (approx. 10 hours).

The trend follows the linear regression until around hour 220 (day 9), where the trend is scattered and becomes random. When the experiment was stopped, the scaffold was torn apart which explains why the bioreactor is no longer able to measure the stiffness of the cell-seeded PGS-M. However, the experiment does show how the stiffness decreased subtly over time, which can be explained if there was a small tear in the scaffold that was gradually torn more over time until the scaffold was torn apart on day 9. In addition, as there was contamination, there was likely low cell action which would increase the stiffness of the scaffold. This reduction in stiffness could also be due to scaffold degrading over the time of the experiment. A future experiment which tests the PGS-M scaffold stiffness against its age in culture conditions would help identify the degradation rate and whether this drop in stiffness is reflective of the degradation of the scaffold. Questions arise as to why the scaffold ripped apart. One explanation could be due to scaffold manufacturing error, another could be due to over-extension of the scaffold during the in vitro experiment and again reflects why it is very important to use mechanical property-specific stimulation parameters based on the scaffold's current material properties. Further in vitro experiments will confirm whether this phenomenon is a one-off error or a recurring problem that will need to be addressed by design intervention.

## 7.5 Chapter Summary

Experiments that explore appropriate in vitro protocols were discussed in this chapter. It is clear that further in vitro testing is needed to confirm the functionality of the robotic bioreactor but these initial experiments show promising preliminary testing of the device. Overall, preliminary in vitro testing is an important step in the development of new tissue engineering tools. It allows researchers to evaluate the safety and efficacy of the tools and optimize their performance. Challenges are commonly faced when working with cells. In the vitro testing reported, the sensor in the Ebers incubator failed. This is unprecedented and as cells rely on several optimum conditions to survive, these failures have a significant impact on the outcome of experiments. Such sensors and regulators that are relied upon, are expensive and time-consuming to fix, often requiring engineering from the supplier to address the problem. The current prototype of the robotic bioreactor uses multiple electrical components which need to perform in an electronically unfavourable environment. Inside an incubator, there is a high humidity level to prevent the cell media from evaporating and leaving the cells without a nutrient supply.

## Chapter 8

## **Conclusions and Future Work**

In this report, the development towards a functional robotic bioreactor was presented and discussed. The development was sectioned into the mechanical design, control design, stiffness measurement comparisons and in vitro protocol. Developing a robot for tissue engineering presents unique challenges due to the complexity of biological systems and the need for precision and accuracy. Tissues in the body are constantly exposed to mechanical forces, such as stretching, compression, and shear stress, which play an important role in regulating cell behaviour, tissue development, and function. Therefore, tissue engineering researchers aim to replicate these forces in bioreactors to create more functional and realistic tissues.

The stiffness of a cell-seeded scaffold can change over time due to various factors such as cell proliferation, extracellular matrix (ECM) production, and degradation. When cells are seeded onto a scaffold, they attach to the scaffold and begin to proliferate and produce ECM. This process can cause the scaffold to stiffen over time, as the cells and ECM build up and create a more rigid structure. Additionally, the stiffness of the scaffold can also be influenced by the mechanical properties of the cells themselves, as well as the surrounding environment. Studies have shown that the stiffness of the scaffold generally increases over time as the cells and ECM build up, and the scaffold becomes more dense and rigid. However, the opposite effect can also occur. As cells degrade the ECM and the scaffold loses some of its structural integrity, the scaffold can become softer and less stiff. This can happen when the scaffold is subjected to mechanical forces or as the result of cellular activities such as the secretion of enzymes that degrade the scaffold material. Therefore, the stiffness of a cell-seeded scaffold is not constant and can change over time depending on the behaviour of the cells and the scaffold properties. Measuring the stiffness of a cell-seeded scaffold at different time points can be important for understanding the scaffold's mechanical properties and how they might influence cell behaviour or tissue development.

Throughout this chapter, bold text will highlight recommendations for future works for the development of the robotic bioreactor.

### 8.1 Prototype Design

A first-generation working prototype of the robotic bioreactor was developed from initial prototypes from previous students in the lab and presented in the first chapter of this thesis. The previous prototype was developed but failed on initial testing. Each individual part of the prototype was refined to improve the performance. The refined first-generation robotic bioreactor was presented at the International Conference on Robotics and Automation (ICRA) 2021 (Appendix B). The assembly consisted of a sterilisable assembly, the chamber and clamps, and a non-sterilisable assembly, the motor, gears and force sensor. There were a few key issues with the first-generation prototype that needed to be addressed with design improvements. The chamber was printed in ABS, which presented problems holding liquid effectively. The force transduction mechanism (hook and compression force sensor) was difficult to set up repeatedly as friction forces affected the ability of the sensor to sense small forces. The worm gear and rack that were used to convert the rotational motion provided by the motor into linear motion had a significant backlash. This meant that for a full rotation in the motor, the translational did not achieve the predicted stroke distance. Inheriting a prototype at the start of the project was both an advantage and a disadvantage. On one side, taking over a project from someone else means that work can build on the existing work, rather than starting from scratch. This can save time and resources. It also presented the opportunity to learn from the previous developer's mistakes, successes, and challenges, and apply these lessons to future projects. On the other hand, the person who originally developed the initial prototype project was initially more familiar with the project's intricacies, design decisions, and limitations than I, who was new to the project. This made it challenging to understand the nuances of the project and difficult to make changes to the prototype early on.

In the design, there were a lot of parts that were easily knocked out of place and affected the alignment of the sensors. A base helped to reduce these movements, but they still existed and it was difficult to achieve the same setup with each experiment.

The second-generation robotic bioreactor was designed to address some of the issues raised with the first-generation design. The material used to print the sterilisable assembly was changed from ABS to dental SG resin, which was UV 3D printed, was 100% and so held liquid much better than ABS printed using FDM. The entire force transduction mechanism was replaced with a single tensile force sensor to reduce the noise created by the force transduction mechanism.

3D printing was used as the main manufacturing process in both prototypes as it allowed for rapid prototyping, which meant that physical prototypes could be made and tested quickly, speeding up the design iteration process. Traditional prototyping methods, such as injection moulding, can be expensive and time-consuming, especially for small production runs. 3D printing, on the other hand, is relatively affordable and can be used for small-scale production. For this project, it was very important to be able to create customized parts and prototypes, tailoring them to the specific needs and requirements of the in vitro experiments. Overall, 3D printing was a powerful tool for prototyping that can help create better prototype designs faster and more cost-effectively than traditional prototyping methods. One disadvantage of using 3D printing to manufacture the robotic bioreactor was that the materials were limited to those capable of being 3D printed. Materials commonly used in vitro experimentation, like glass and metal, are not commonly 3D printed and this makes the process of sterilisation difficult and less commonly used sterilisation methods must be explored. 3D printers also limit the size of the objects they can produce, which wasn't a huge limitation in this project but should be considered when moving on to different uses of the robotic bioreactor. Once the bioreactor has been extensively tested, the next step would be to outsource the manufacturing of the robotic bioreactor. Changing the manufacturing techniques would allow the chamber to be manufactured from materials that are commonly sterilised via autoclaving which was a persistent problem in the project.

The development of position and force control was presented and compared. Motor encoders and force sensors are two types of sensors commonly used in robotics and mechatronics applications and both are key to the function of the robotic bioreactor. While both types of sensors provide important information for controlling robotic systems, they have different capabilities and limitations when it comes to accuracy. Position control used the position from the motor sensor, ie the magnetic encoder on the DC motor, to control the displacement of the cell-seeded scaffold. For force control, the force sensed by the force sensor was used as the control signal to control the displacement of the cell-seeded scaffold.

Although both types of control were capable of providing mechanical stimulation, the two control types rely on totally different feedback signals and therefore are highly dependent on the specifications of the sensor used in the design. As force sensors and motor encoders measure different physical quantities and are subject to different sources of noise. Typically, the force sensor was subject to more noise and therefore provided a less stable control feedback signal. So with the current prototypes, position control was more suitable and reliable as the control method. However, future iterations with higher-quality force sensors could provide the necessary accuracy and stability to perform as the feedback signal. Such force sensors have significantly higher costs in comparison to the motor encoders available and should be considered.

## 8.2 Performance Comparison

To quantify if the changes made in the second-generation bioreactor were beneficial, the two prototype designs were compared in three key areas; displacement accuracy, force measurement accuracy and the ability of the chamber to hold liquid without leaking.

Displacement accuracy affects the mechanical stimuli experienced by the cells. The displacement accuracy of a bioreactor determines the magnitude and frequency of mechanical stimuli experienced by the cells in the engineered tissue. These mechanical stimuli are essential for maintaining the tissue's structural and functional integrity and influencing its development and maturation. It also facilitates the optimization of bioreactor parameters. Accurate displacement measurement enables researchers to optimize bioreactor parameters, such as the frequency and magnitude of mechanical stimuli, to maximize tissue growth and development. In addition, more precise and accurate displacement measurements enhance the reproducibility of tissue engineering experiments. Accurate displacement measurement can increase the reproducibility of tissue engineering experiments by ensuring that the same mechanical stimuli are applied consistently to different batches of tissue.

The accuracy of the robotic bioreactor displacement measurements was determined using a webcam tracking protocol. The movements of the clamps were recorded and tracked using tracking software. The DC motor used in the first-generation robotic bioreactor was compared to the linear actuator used in the second-generation robotic bioreactor. The relative error for both motors was found to be similar, however, the standard deviation for the linear actuator was much smaller. A smaller standard deviation indicates that the data points in a set are closer together, meaning they are more tightly clustered around the mean or average. In the context of the robotic bioreactor, this is considered better because it suggests that the data points are more consistent and less variable. In the context of displacement measurements, a small standard deviation indicates that the measurements are more precise and consistent.

The robotic bioreactor action will be based on stiffness measurements over time. The stiffness measured will be calculated using displacement measurements and force measurements obtained from the robotic bioreactor. Both sensor measurements must be accurate and precise to calculate a stiffness reflective of the true material's mechanical properties.

From the experiment where force measurements were compared between the first and second-generation bioreactor force sensors, it was clear that both sensor gave high variation which can be explained by the sensors being downstream of the source of the force. Future designs to change the position of the sensor could be explored and the impact this would have on the measurement accuracy. Moving the force sensor within the chamber would provide closer measurements but presents challenges such as how to keep the force sensor sterile when it cannot be autoclaved or submerged in IPA. Options to isolate the force sensor using a membrane could also be explored but might complicate the set-up of the system prior to in vitro experiments.

Further studies into the impact of the PP chamber membrane on the force measurements could also be explored. Specifically, whether the variation in setting up the membrane could impact the force measurements as an additional source of interference. By also testing the pre-tension regime thoroughly, it might help explain the force sensor variation, as if the pretensioning of the scaffold varies from each experiment then the starting point of the experiment changes.

As discussed earlier, the lower forces are difficult to sense and using the force sensor to pre-tense the scaffold may prove to be unreliable. However, as the scaffold is clamped under a biosafety cabinet, it is also difficult to clamp the scaffold precisely each time resulting in different areas of the scaffold being clamped in different experiments. In addition, as clamping the scaffold is a human input, this would not only differ between experiments by the same user due to human error, but it would also change between users due to different techniques and experiences. Future iterations of the robotic bioreactor could include 'tensioning' screws to enable the user to tension the scaffold manually with greater precision that can be achieved by the motor. More sensors could be used to cross-reference measurements but this adds complexity to the system which may cause problems during long-term experiments. There may be scope to use the current drawn through the motor to crossreference force readings and explore whether there is a relationship between the force and the current drawn from the motor.

It is important to seal the chamber of a tissue engineering bioreactor to maintain a controlled and sterile environment within the bioreactor. It helps to prevent contamination. Sealing the chamber helps to prevent microorganisms or other contaminants from entering the bioreactor and potentially contaminating the tissue culture. This is especially important for long-term cultures, which are more susceptible to contamination. Ensuring an effective seal around the chamber helps to minimize evaporation of the culture media, which can affect the nutrient and waste exchange in the tissue culture. This can impact the viability and function of the tissue developed in the bioreactor.

The first-generation and second-generation membrane designs were compared on their ability to hold liquid over time. The second-generation robotic bioreactor membrane design showed no signs of leaking after a twenty-four-hour experiment, whereas there was significant leaking from the first-generation membrane design. The two main parts of the membrane design were then isolated and tested at their extreme where there were no visible signs of leaks. These types of extreme testing are ideal for checking the effectiveness of the membrane prior to in vitro experiments. This reduces the risk of unexpected problems occurring during in vitro experiments. Overall, sealing the chamber of a tissue engineering bioreactor is essential for maintaining a controlled and sterile environment within the bioreactor. This can help to optimize tissue growth, development, and function, and increase the reproducibility of tissue engineering experiments. Further tests need to be conducted to determine whether the lid effectively seals the chamber. Simple tests such as filling the chamber with water, sealing the lid and then flipping it upside down and checking whether any water leaks out.

### 8.3 Using stiffness to track cell development

Tracking the development of a cell-seeded scaffold is very challenging. Tissue development is a complex process that involves many factors, including cell proliferation, migration, differentiation, and extracellular matrix deposition. Cells are highly heterogeneous and can behave differently depending on their origin, environment, and interactions with other cells. This heterogeneity can make it challenging to accurately track the behaviour of cells seeded onto a scaffold. Cell behaviour is also highly dynamic and can change rapidly in response to changes in their environment. For example, cells may respond differently to changes in nutrient or oxygen levels or changes in mechanical forces and these changes can be difficult to predict and monitor. Imaging techniques used to track the development of cell-seeded scaffolds, such as confocal microscopy or histology, can be time-consuming, require specialized equipment, and may not provide real-time information.

Using the stiffness of a cell-seeded scaffold has the potential to provide real-time, non-destructive monitoring of tissue construct development. Mechanical properties, including stiffness, are critical for tissue functionality, as they dictate how tissues respond to external forces. By tracking the stiffness of a cell-seeded scaffold, researchers can gain insight into how the cells on the scaffold develop over time, which could provide vital insight into cell action during development. This could also show how the scaffold will behave in the body, should it be implanted, and whether it will be able to support the formation of new tissue. A huge issue with tissue-engineered tissue is that tissue-engineered tissues often lack the mechanical strength and stiffness required for certain applications, such as load-bearing tissues. The ability to track how the tissue develops over time can give vital information as to whether scaffolds can be used to support tissue growth in vivo and provide the appropriate mechanical properties during tissue development. The mechanical properties of a scaffold can also affect the behaviour of cells seeded onto it. For example, cells will respond differently to a scaffold with a high stiffness versus a low stiffness. By tracking the stiffness of the scaffold, researchers can monitor how cells are responding to their environment and how they are influencing the mechanical properties of the scaffold over time. As the stiffness measurements can be non-destructive, meaning that the scaffold can be repeatedly tested without damaging it. This is particularly important for long-term studies where multiple measurements over time are required to track the transitional changes in the mechanical properties of the scaffold, rather than just the end-state stiffness.

However, these stiffness measurements require the two sensors in the robotic bioreactor to be highly accurate and precise in order to provide accurate stiffness information. Materials can exhibit nonlinear behaviour, meaning that the relationship between stress and strain is not always constant. This can make it challenging to accurately measure stiffness because the stiffness may vary depending on the magnitude of the stress or strain. This in particular highlights the importance of selecting the most appropriate area to measure the stiffness.

Another major challenge with using stiffness measurements to track a cell-seeded scaffold development is that tissues are typically non-homogeneous and have different stiffness properties in different parts of the tissue. This can make it difficult to obtain an accurate measurement of the overall stiffness. The stiffness of a material can also be affected by environmental factors such as temperature, humidity, and time. These factors can make it challenging to obtain consistent and accurate measurements. Proper preparation of the sample can also be critical to obtaining accurate stiffness measurements. The size and shape of the sample can impact the stiffness measurement, and the sample must be prepared in a way that minimizes any variability in the stiffness properties. This is challenging when working with cells, as scaffolds used in tissue engineering can have variable properties due to differences in manufacturing, storage, or handling. These differences can affect cell behaviour and tissue formation, making it difficult to achieve consistent results across multiple experiments.

For the application of using stiffness to base the robotic bioreactor's control, the question arises as to how accurate the stiffness measurement needs to be to provide sufficient information to use for a control system. It is highly dependent on the type of tissue and scaffold used in the experiment. Accurate stiffness measurements are important for both low-stiffness and high-stiffness materials, but they may be more critical for low-stiffness materials for several reasons. In this context, low stiffness refers to scaffolds and cells that work towards developing into a low-stiffness tissue, such as adipose tissue, and high stiffness refers to scaffolds and cells developing into high-stiffness is likely to be very low and will therefore require highly accurate and precise stiffness measurements to be able to track the stiffness changes accurately

as they are likely to be small and subtle. Whereas, low stiffness materials have a low Young's modulus, which means that they are more susceptible to deformation and stress at low loads than higher-stiffness materials. This makes accurate stiffness measurements more critical for low-stiffness materials as even small errors in measurement can lead to significant deformation. For these lower-stiffness tissues, other nondestructive methods of tracking the development of the cell-seeded scaffold may be more appropriate.

Experiments were conducted to determine the most appropriate methods of stiffness computation over a range of difficult conditions. These results showed. not only that the stiffness computation accuracy was depending on the area of the waveform used for computation but also on how it varied depending on the stimulation regime used. The stiffness computation methods were also limited to sinusoidal waveforms, which reduces the versatility of the platform. Sinusoidal displacement waveforms introduce dynamic effects in the system. These dynamic effects can include resonances, vibrations, and inertial forces that can influence the measured displacement. These additional factors can complicate the interpretation of the measured data and make it challenging to isolate the stiffness component accurately. By including a stiffness computation programme that is independent of the stimulation regime executed in an in vitro experiment, i.e. a ramp signal that is executed every time a stiffness measurement was taken, this could provide more stable, repeatable stiffness measurements which are also unaffected by the stimulation regime used in the experiment.

In the stiffness comparison experiments, the stiffness was computed at every time step during a specific part of the waveform and then averaged. Future experiments could also look into using the difference between two stiffness value formulas,  $\frac{(f_1-f_2)}{(d_1-d_2)}$ . By measuring stiffness at different levels of force and displacement, it is possible to determine whether a material exhibits linear or non-linear behaviour. Linear materials exhibit a constant stiffness regardless of the level of force applied, while non-linear materials exhibit a change in stiffness at different levels of force or displacement. Measuring stiffness across a range of forces and displacements can help to identify these non-linearities and provide a more accurate understanding of the material's behaviour.

How often the stiffness measurements should be captured is also a key area that needs to be further investigated. As shown in Sec. 6.2 arbitrarily selecting a frequency to measure stiffness may not capture the changing stiffness sufficiently. This frequency will need to be adjusted for different scaffolds and cells used in vitro. In some cases, measurements may need to be taken continuously in real-time to detect rapid changes in mechanical properties. In other cases, measurements may be taken at regular intervals. **This could be something that is coded into the robotic bioreactor, where if**  a change in stiffness occurs that is considered significant, ie falls outside the error boundaries, stiffness measurements could be taken more frequently to determine whether the stiffness change was accurate or an error and also to capture any further immediate changes in stiffness. These stiffness measurements can not only enable adapted stimulation regimes but can also help identify potential issues early and take steps to prevent material failure or other issues.

The next step to developing the stiffness-based control should be identifying the limits and proportions of the control implemented. For a specific increase in stiffness, a proportional increase in stimulation should be implemented. This becomes very challenging to tune, as a change in stimulation that provide too much stimulation could damage the scaffold but a change in stimulation that does not provide enough stimulation isn't providing anything more than commercial bioreactors' open-loop stimulation strategies. This in itself is an interesting research area and could be its own project in itself and lead to some exciting research into optimising stimulation regimes over a changing tissue construct. Previously changing the extension percentage has been discussed but also sinusoidal frequency could be altered depending on the stiffness of the tissue construct.

### 8.4 In vitro protocols

Further in vitro experimentation would help inform the development of the stiffnessbased control. Problems that arose from the in vitro experiments could be better investigated by isolating parts of the robotic bioreactor for experiments. One future in vitro experiment could use a chamber from a commercial bioreactor which has been already approved. This could allow further validation of the robotic bioreactor stimulation behaviour isolated from the chamber, whilst further sterilisation investigation continues. One experiment that could be used to compare the robotic bioreactor action would be to conduct an experiment where the stiffness of a cell-seeded scaffold is stimulated using a commercial bioreactor and the stiffness is destructively tracked throughout an experiment. This would enable reliable tracking of the stiffness development of a cell-seeded scaffold to compare the results of the robotic bioreactor. This experiment would, however, require multiple experiments running simultaneously with multiple cell-seeded scaffolds that could be destroyed at specific intervals. For example, twelve cell-seeded scaffolds are simulated the same, and three are tensile tested and the stiffness is logged after seven days, fourteen days, twenty-one days and twenty-eight days. This allows the stiffness of the cellseeded scaffolds to be compared at different time stamps in an experiment. This experiment would be time-consuming and is the exact experiment the robotic bioreactor could replace once it is fully functional.

This sterilisation investigation would involve making the necessary steps to allow the chamber and counterparts to be sterilised. This would include replacing all the acrylic parts, including the membrane washers and the lid, with a material that can withstand the autoclaving conditions without material deformations, like glass or metals, and also reinforcing the places of the chamber which cracked during the autoclaving.

At this point, it is difficult to draw comparisons with commercially available bioreactors. However, commercially available bioreactors usually have a set stimulation regime and limit experimentation to these regimes. The bioreactor can achieve a range of stimulation regimes of various amplitudes and can be fine-tuned for any experiment, making it a much more versatile tool for tissue engineering.

The cost of a tissue engineering bioreactor can vary widely depending on the specific requirements of the application, the type of bioreactor, and the manufacturer. A basic laboratory-scale bioreactor can cost anywhere from a few thousand to tens of thousands of pounds, while a larger, more sophisticated bioreactor system used for commercial-scale production can cost hundreds of thousands to millions of pounds. The cost breakdown summarised in A.1 and A.2 shows that the bioreactors developed in this project are significantly cheaper than those that can be purchased commercially. Even if the parts that are 3D printed were outsourced and machined, the cost would still be low.

Factors that can affect the cost of a tissue engineering bioreactor include the size and complexity of the system, the types of sensors and control systems used, the materials used in construction, and the level of automation and customization required. Keeping the bioreactor simple, ie one motor sensor and one force sensor helps to keep the costs down significantly. It is important to note that the bioreactor cost is just one component of the overall cost of tissue engineering, which includes the cost of biomaterials, cell culture reagents, labour, and other resources required to develop functional tissues.

There are also a number of hidden costs when manufacturing and commercially selling a tissue engineering bioreactor. They require rigorous validation and testing to ensure that the system meets the required standards for safety and efficacy. Developing a bioreactor in-house, without the intention of commercially selling it, to explore new areas of inquiry, make more precise measurements, and develop more effective stimulation regimes that cannot be achieved with modern tissue engineering bioreactors.

Many of the commercial bioreactors available are mostly rigid-bodied and used tradi-

tional components. The movement towards softer designs of bioreactors could unlock new possibilities when engineering tissue in vitro. Soft materials are better able to mimic the mechanical properties of natural tissues, such as elasticity, compliance, and deformability. This allows for more accurate replication of the in vivo environment, which can improve tissue development and maturation. Soft materials can also provide a more favourable environment for cell adhesion and proliferation. which is critical for tissue growth and development. The flexible and porous nature of soft materials allows for better nutrient and oxygen transport to the cells, resulting in improved cell viability and functionality. Shear stress is a common problem in traditional bioreactors, which can lead to cell damage and reduced tissue quality. Soft material-based bioreactors can reduce shear stress due to their lower stiffness and the ability to deform under mechanical loading. Soft material-based bioreactors are more flexible and adaptable than traditional bioreactors, making them easier to customize and scale up. They can be easily shaped into different geometries and sizes to accommodate various tissue types and applications. Overall, soft material-based tissue engineering bioreactors have the potential to improve tissue engineering outcomes and enable the development of more complex and functional tissue constructs.

### 8.5 Summary

Developing a robot for tissue engineering requires a multidisciplinary approach, combining knowledge from mechanical, electrical, and software engineering, as well as biology and chemistry. It also requires close collaboration between engineers, biologists, and clinicians to ensure that the system meets the specific requirements of tissue engineering applications. Developing new tools is critical for advancing scientific understanding, addressing complex research questions, increasing efficiency and accuracy, developing new treatments, and stimulating innovation and collaboration.

Robotic techniques, used to design and control advanced bioreactors, can provide more precise and complex mechanical and chemical signals to engineered tissues. This could help to optimize tissue formation and maturation, and potentially accelerate the development of functional tissues for transplantation. Examples of robotics used to develop in situ tissue engineering approaches are becoming more common in literature, where tissue engineering materials and devices can be implanted directly into the body and then controlled and monitored using robotics. This approach could potentially enable the regeneration of damaged tissues and organs without the need for transplantation.

Overall, the future of robotics in tissue engineering is likely to involve the integration of robotic technologies with existing tissue engineering techniques to create more efficient and effective approaches for creating functional tissues for a variety of applications, including regenerative medicine, drug discovery, and disease modelling.

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Appendices

# Appendix A

# Appendix A

Part	Vendor	Cost (f)	Description
Chamber	Plot IT	53.00	ABS 3D printing material
Clamps	Plot IT	16.00	ABS 3D printing material
A4 stainless	Accu	1.02	Used to secure clamps
steel nuts			
A4 stainless	Accu	1.62	Used to secure clamps
steel bolts			
Compression	Honeywell	66.56	Force sensor
Force sensor			
Force Sensor	Texas	8.18	INA121P
Amplifier	Instruments		
Force	Plot IT	12.00	ABS 3D printing material
Transduction			
Mechanism			
Worm Gear and	Plot IT	21.00	ABS 3D printing material
Rack			
Motor housing unit	Plot IT	8.00	ABS 3D printing material
Dual Shaft DC	Pololu	23.65	Used to drive clamp
Motor			movement
Magnetic	Pololu	8.02	Used to sensor position
encoder			of clamps
Base	RS	10.00	PLA 3D printing filament
Arduino Nano	RS	17.86	Microcontroller
Other	RS	25.00	Prototype board,
electronics			wires etc
	Total Cost	271.91	

 Table A.1: Cost breakdown of the first-generation bioreactor

Part	Vendor	Cost (f)	Description
Chamber	Formlabs	68.00	Dental SG resin
Clamps	Formlabs	23.00	Dental SG resin
A4 stainless	Accu	1.02	Used to secure clamps
steel nuts			
A4 stainless	Accu	1.62	Used to secure clamps
steel bolts			
Force sensor	Loadcell	279.90	Low Capacity Load Cell
& 0-5V Module	Shop		
Linear Actuator	RS	113.87	Actuonix L12-10-210-12-P
Base	RS	7.00	PLA 3D printing filament
Arduino Nano	RS	17.86	Microcontroller
Other electronics	RS	30.00	Prototype board, wires etc
	Total Cost	542.27	

 Table A.2: Cost breakdown of the second-generation bioreactor



**Figure A.1:** Force transduction mechanism with two compression force sensor, where force sensor A measures compressing forces and force sensor B measures tensile forces. a) Side-view of the two compression force sensors, b) Top-view of the two compression force sensors and c) side-view illustration of force transduction mechanism.



**Figure A.2:** Displacement and force data obtained from the robotic bioreactor using the two compression force sensors. Top plot shows the displacement of the motor in steps to 5mm and back to 0mm. The middle plot shows the force data obtained from force sensor A. The bottom plot shows the force data obtained from force sensor B.



**Figure A.3:** Force tensioning flow diagram describing the steps programmed into the bioreactor to decide whether to execute the force tensioning programme and when to stop. The grey blocks represent actions taken by the bioreactor and the blue boxes represent



Figure A.4: MATLAB GUI created for the robotic bioreactor

# Appendix B

# Paper 1

# Design and Development of a Robotic Bioreactor for In Vitro Tissue Engineering

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Abstract-In this study, a novel robotic bioreactor is presented with capabilities of closed-loop control of force and displacement applied to a tissue scaffold and tissue scaffold stiffness calculation. These characteristics bring the potential of a robotic bioreactor that can optimize the mechanical properties of tissue constructs in order for them to match those of native tissues. Custom position and force control signals are designed to maintain a steady tensioning of the tissue scaffold while the latter one's mechanical properties evolve in time. We propose a simple model to support the hypothesis that the stiffness of a cell-seeded scaffold increases over time, and thus force control signals need to be adjusted accordingly. The robotic bioreactor is able to measure the stiffness of a scaffold sample relatively accurately, with an average standard deviation of 0.2N/mm. The combination of accurate stiffness measurements and a closed-loop control system equips the robotic bioreactor with the fundamental requirements to achieve stiffness based force control in future in vitro experiments, and thus to a tissuescaffold responsive technology for advanced tissue engineering.

#### I. INTRODUCTION

*In vitro* testing offers a platform to perform more detailed analysis of cells or tissues that would not be possible with a whole organism, whilst also having the ethical advantage of avoiding animal testing. It can help explain, with greater detail, isolated behaviour of tissue or cells and form a clearer picture into how that behaviour contributes to the organism. *In vitro* testing can also determine testing conditions for later *in vivo* experiments, such as with implantable devices.

Cells respond to mechanical stimuli [1]. The stimuli can induce proliferation, differentiation, apoptosis, or secretion of extracellular matrix (ECM) deposition [2]. This mechanostimulation can be taken advantage of to develop therapies, such as novel robotic implants that apply tension to esophageal tissue to encourage cell growth and reconstruct missing tissue areas of the organ [3]. Research into optimum mechanostimulation regimes are vast and suggest that different regimes induce different results. The types of cell and environment used can also affect the results of a study, including cell proliferation capability and ECM production [4][5]. All these variables, however, require long and time consuming iterative experiments in search of globally

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Fig. 1. Robotic bioreactor. a) Illustration of the protocol that will be followed to validate the bioreactor in an *in vitro* experiment. b) Image of the robotic bioreactor.

optimum conditions, as current tissue engineering tools are limited by a lack of sensing and adaptive control methods.

#### **II. RELATED WORK**

While tissue engineering has great potential in constructing tissues and organs from scratch, there are multiple challenges recognised in the field, including identifying the optimum protocol to stimulate a population of cells so they develop in a way that recreates the geometrical and mechanical properties of the target native tissue [6]. Bioreactors are used in tissue engineering to mimic and regulate the environment of the cells during *in vitro* experimentation. Cells are seeded for mechanical support in a biomaterial and are bathed in a nutrient medium. Mechanical stimulation is applied either through tensioning, pressure or fluidic shear. Commerical bioreactors that induce mechanical stimulation, such as Ebers TC-3 Bioreactor [7], BioTense [8], Cartigen [9], are regularly used in tissue engineering. Results from experiments using commercial bioreactors have shown increases in extracellular matrix (ECM) deposition and cell proliferation [4][10][11]. The ECM is a non-cellular complex network that provides structural support to the cells and determines the stiffness of the tissue [12].

Other research groups have also developed their own bioreactor to suit the needs of their experiments. In particular, in [13], a bioreactor utilizes force sensors in its design to monitor the force throughout experiments. They do not, however, use this force data to alter the stimulation applied to the cell-seeded scaffold (CSS) during the experiment. With most bioreactors, including the ones previously mentioned, once the program has been set, there is no change to the programme during the experiment. This means there is no recognition that the global mechanical properties of the system changes over time, leading to the research question: if the global system is changing its own mechanical properties, should the mechanical stimulation applied to the scaffold also adapt?

The robotic bioreactor design proposed in this paper, focuses on the application of tissue-engineering fundamentals into an autonomous robot to encourage the creation of a different tissue which resembles native tissue on a CSS. The motivation for this study comes from the hypothesis that if a CSS is changing its mechanical properties throughout the experiment, then a force required to deliver a true constant mechanical stimulation also changes.

The aim of this paper is to present the theoretical and practical benchtop validation of a 3D printed robotic bioreactor. The study will introduce the fundamental requirements to enable stiffness-based force control in *in vitro* experiments in order to achieve optimisation of the mechanical properties of tissue constructs. The contributions of this paper are (1) the design and development of a novel robotic bioreactor with scaffold tension and displacement sensing; (2) the development towards custom stiffness-adaptive force and position control; (3) the proposal of a future protocol needed to validate the bioreactor *in vitro*.

# **III. MECHANICAL DESIGN**

The robotic bioreactor consists of mainly two parts: (1) a sterilizable assembly where the cells are kept in biological conditions, and (2) a non-sterilizable assembly dedicated to the sensing of the CSS and control of the robotic bioreactor. This design allows the merging of a typical protocol of *in vitro* cell seeding and a post-seeding control setup.

# A. Sterilizable Assembly

1) Chamber: The foundation of any bioreactor is that the cells should be able to survive and thrive within the chamber and so a sterile environment must be maintained. The chamber is fabricated with Acrylonitrile butadiene styrene



Fig. 2. CAD design of the robotic bioreactor. a) Main components of the robotic bioreactor; b) Details of the robotic bioreactor clamps. The clamp teeth grip the scaffold and hold it in place whilst mechanical stimulation is applied. They are engaged by tightening the bolts at the top of the clamp.

(ABS) using a fused deposition modeling (FDM) 3D printing technique. The chamber measures 116mm × 55mm × 88mm, with a lid sealing the chamber measuring 120mm × 105mm laser cut from 6mm thick acrylic sheet. Furthermore, during *in vitro* experiments clean sterile air needs to be pumped inside the chamber and constantly recycled during the experiment. A 0.22  $\mu m$  filter is used to facilitate gas transfer between incubator and the cell culture environment. To accommodate this filter, a hole was laser cut in the lid (Fig.3a). The filter would be added under the class 2 biological cabinet after the chamber and its counterparts have been sterilized.

Cell medium is added into the chamber to encourage and maintain cell survival, growth and replication whilst preventing contamination. In order to make the chamber water impermeable, the inside of the chamber is melted with acetone. A custom sealing ring is fabricated from Ecoflex 00-30 and placed on the top margins of the chamber to ensure that the lid is sealed and air tight. Four nuts and bolts are used to secure the lid at the four corners of the chamber.

2) Clamps: The CSS is held by two ABS clamps produced by rapid prototyping and feature teething patterns for a secure grip of the scaffold as shown in Fig.2b. While one clamp is fixed to the wall of the chamber with screws (distal clamp), the other one is driven by the external control module along a linear direction to pull and relax the scaffold (proximal clamp). The clamps, not including the rod which is attached to the clamp (proximal), measures  $35\text{mm} \times 38\text{mm}$   $\times$  18mm. The moving clamp (proximal) is supported by the chamber floor which as designed with built-in tracks. These tracks inhibit the lateral movement of the clamp, which is likely to damage and tear the scaffold, while minimising the friction between the two elements. The clamp teeth are designed to grip the scaffold evenly to distribute the force across the scaffold as localised force is likely to damage the scaffold. The process of clamping the CSS is performed under a class 2 biological cabinet. This has contributed significantly to the design of the clamps so that the clamping can be performed using only sterilized tools to reduce the risk of contamination to the chamber and CCS.



Fig. 3. Details of the robotic bioreactor a) The chamber. A membrane keeps the chamber water tight and prevents contaminants to enter the sterile chamber, which is kept in place by the rob sealing screw and chamber sealing screw. The hole in the lid is to accommodate for the air filter during *in vitro* experiments. b) Force sensor mounting mechanics. The force sensor is soldered onto a breadboard and then glued to the force sensor housing so that the 'hook' of the rack engages with the force sensor when the scaffold is tensioned. The rack stopper is used to stop the hook misaligning with the force sensor.

3) Sealing membrane: The clamp (proximal) exits the chamber through a hole in the chamber wall, which creates an entrance for contaminants to enter from the external environment into the chamber. It is crucial to isolate the chamber from the external environment. This has been done by using a separating polyurethane membrane of 0.4mm thickness (Fig. 3a). The membrane is attached to the outside of the chamber using a thread and a counterpart to the thread that tightly screws around the membrane to maintain a strong grip against the junction. The second part of the membrane is attached to the rod using the same threading and screw mechanism. The membrane has been tested successfully against leakage by filling the chamber with medium (pink color), leaving it for 3 days and visually surveying for any leaks.

# B. Non-sterilizable Assembly

The actuation module is placed outside of the sterile chamber and encompasses electronic components that would not withstand the sterilization process required for the chamber. The actuation module enables the linear displacement of the proximal clamp in the chamber. A DC-motor-driven worm gear, with a pitch of 1.95mm, meshes to a teethed rod which is connected to the proximal clamp. This module is placed outside of the chamber.

A mechanism was designed to accommodate a compression force sensor that can detect the amount of force applied to the scaffold through each pulling cycle. This sensor was soldered to a protoboard and then was placed and glued between the hook of the teethed rack and the rod of the proximal clamp (Fig.3b).

The base of the robotic bioreactor features tracks to avoid lateral deflections during the linear motion of the rod. The bioreactor and base fit into an area measuring 343mm  $\times$  110mm  $\times$  63mm, so will fit in most conventional incubators that provide the required environmental conditions for cell survival.

# IV. ELECTRICAL DESIGN

The mechanical stimulation of the scaffold is driven by a 12V DC microgear motor (Pololu) with a magnetic encoder placed at the back of the rotor. The motor has a 297.92:1 gear ratio which actuates the worm-gear mechanism. This actuation chain implies an important ratio r between one motor rotation (sensed by the encoder) and an actual rack translation. We can calculate that one step of the encoder senses a 0.00056mm moving of the clamps. From calibration we measure a 0.0006mm movement of the rack from one step in the encoder. The relative error of this ratio is  $e = \frac{|r_{exp} - r_{th}|}{2} = 9\%$ .

A force sensor (Honeywell FSS005WNGT) is used in combination with an amplifier (INA121P), which has a sensing range of 0N to 5N. The force sensor was sampled every 25ms. The force sensor was calibrated with calibrated weights. The controller is implemented on an Arduino MEGA powered from a laptop. Since the sensor is the connection point between the upstream section of the actuation chain (i.e. the motor) and the rod of the clamp that is pulling the scaffold, the force sensed is approximately equal to the actual force applied onto the scaffold. Friction forces do exist between the moving elements of the bioreactor and only contribute to a minor difference between the actual force applied to the scaffolds and the sensed force.

#### V. CONTROL

#### A. Cell-Seeded Scaffold Evolution

The CSS and its material properties evolve in time and the resulting material properties are the consequence of a combination of scaffold degradation, cell proliferation and ECM deposition [2][14][15]. If a scaffold is biodegradable, its mass will reduce along with its material properties over time [16]. At the same time, ECM is being deposited by the cells which contributes to new material properties of the system, such as tensile strength and stiffness [14]. In this study, we consider scaffold degradation and collagen production to mainly contribute to the stiffness of the global system. For future in vitro study, Poly(glycerol sebacate)-Methacrylate (PGSM) will be used as the scaffold. PGSM is a photocurable Poly(glycerol sebacate) (PGS) produced by functionalization with methacrylate groups [16]. PGSM exhibits linear degradation over time at a rate of 20% mass loss at 30 days [16][17]. We assume that a loss of mass of the scaffold will affect material properties, such as a reduction in tensile strength and stiffness. It is therefore predicted that the stiffness is likely to reduce 4.7% over a week long experiment. In [2], experimental results show that cells seeded on PGSM scaffold secreted 50% more collagen than cells growing in 6 well tissue culture plastic plates (control) over a 7 day in vitro experiment. ECM determines the stiffness of tissue [12]. Given that we predict a relatively small change in the stiffness of PGSM, we hypothesise that it will have a small impact on the overall stiffness of the CSS at the end of a 7-day experiment. As collagen is found in the ECM, the increase in collagen also indicates an increase in overall ECM deposition and will result in an overall increase of stiffness of the tissue scaffold. To stimulate the cells in a three-dimensional environment, the force needs to be large enough for the cells to sense and react to it [18] and if the global stiffness increases, as we predict it to, the cells will require a greater force to sense the same mechanostimulation signal throughout the experiment.

#### B. Scaffold mechanostimulation control

1) Position and force control: While typical bioreactors have predefined open loop control, due to changes that occur in the CSS, it is imperative to have a cue of the evolution of the scaffold and be able to adjust the control signals accordingly. For the position control, a sinusoidal target waveform is set with a frequency of 0.25Hz, an amplitude of 3mm and an offset of 3mm to achieve maximum displacement of 6mm and minimum displacement of 0mm. The initial position of the clamps were remotely changed with buttons to control the motor to ensure that the scaffold was pre-tensioned the same amount in each trial.

For force control, a sinusoidal target waveform is set, with a frequency of 0.25Hz, an increasing amplitude starting at 0.4N and an offset that also increases at the same rate at the amplitude to keep the reference signal above 0N. Frequencies of 0.1Hz to 1Hz have been used to stimulate fibroblasts in previous studies, so we chose 0.25Hz for our reference signal to sit within this range [19][20][5]. Both the position control and force control was implemented in Arduino using a PD controller and tuned accordingly. The force sensor readings were filtered with a moving average filter in post processing. A phantom scaffold, fabricated from Ecoflex 00-30, measuring  $30\text{mm} \times 10\text{mm} \times 4\text{mm}$  was used in both the position and force control experiments.

2) Stiffness calculation: Previously, it was discussed that the stiffness of the global system is likely to increase over time. If the stiffness of the CSS is computed at regular intervals throughout a 7-day experiment, we can track how the CSS is changing over time. This parameter can also be used to control the force applied to the scaffold. As the CSS stiffens, a greater force is needed to maintain a constant mechanostimulation. The stiffness of the cell-seeded scaffold can be calculated as:

$$S_s = \frac{F_s}{E_s} \tag{1}$$

where  $S_s$  is the stiffness of scaffold,  $F_s$  is the force on scaffold and  $E_s$  is the elongation of scaffold, as transduced by the sensors in the system.

The stress-strain relationship of the phantom scaffolds was obtained with a uniaxial tensile test on an electric static testing instrument (MX2, IMADA). Two samples of Ecoflex (Ecoflex 00-10 and Ecoflex 00-30) were prepared, measuring 30mm  $\times$  10mm  $\times$  4mm and one sample of PGSM, measuring  $10\text{mm} \times 25\text{mm} \times 1\text{mm}$ . Both Ecoflex samples were pulled at a constant speed of 1 mm/sec and the PGSM sample was pulled at a rate of 0.7mm/sec. The Ecoflex samples were pulled to 15mm displacement to ensure we test within the elastic region of the Ecoflex. The PGSM was pulled to 3.5mm displacement, beyond which its breaking point is reached. Each of the Ecoflex samples was tested three times, first with the bioreactor and then with the IMADA. One trial was conducted with PGSM sample. Force measurements for both the IMADA and bioreactor were filtered using a moving average in post processing.

### VI. EXPERIMENTS AND RESULTS

We tested the capability of the robotic bioreactor to control the displacement and force applied to tissue scaffolds, as well as validate the stiffness calculation. These tests were conducted without cells and using materials such as elastomers as proxy for the scaffold, as well as a biomaterial that is eligible for in vitro tissue engineering.

A. Position and Force control



Fig. 4. Displacement control of the scaffold. (Top) The position control signal (red) shows the desired trajectory of the clamp displacement compared to the actual displacement of the clamps (blue). (Bottom) Force sensor readings during position control experiment.

In Fig. 4, the desired trajectory of the position control is shown as a sinusoidal waveform. Following the peaks in the



Fig. 5. Force control of the scaffold. (Top) The force control signal (red) shows the desired trajectory of the force measured by the force sensor compared to the actual force readings (blue). (Bottom) Displacement of the clamps from their original position during force control experiment.

force sensor reading, a sharp drop in force is shown. This is expected as the force sensor is only being compressed when the clamp is pulling the scaffold in tension. When the clamp direction is reversed, there are no compression forces acting on the force sensor. The secondary peaks that occur at approximately 7 and 11 seconds are likely a result of the combination of the viscoelastic behaviour of the phantom scaffold and sensitivity of the force sensor.

In Fig. 5, we show a desired force trajectory compared to the actual force measured by the force sensor. We see a general trend of the force sensor reading following the trajectory and the position of the rack in response to the force control. The amplitude of the target force signal increases over time which is achieved by increasing the displacement of the scaffold until the desired force is achieved. The behaviour demonstrates the potential behaviour of stiffness-based force control; when the stiffness of the CSS increases over time, the force applied to the scaffold also increases so that a constant force is applied to the scaffold throughout the experiment.

## B. Stiffness Calculation

1) Stiffness calculation validation with tensile test machine: Fig. 6 shows that the bioreactor measurements follow the trend of the tensile testing machine with the average standard deviation of the mean for the bioreactor measurement calculated as 0.10N/mm for Ecoflex 00-10 and 0.37N/mm for Ecoflex 00-30.

Fig. 7 shows the clamps following a ramp trajectory, until the scaffold has been displaced 3.5mm. At 14% stretch, the PGSM reaches its tensile limit. This is shown by the sharp increase in force at 3.3mm displacement.

2) Stiffness derivation during mechanostimulation regimen: In this experiment, the stiffness calculation process for long-term *in vitro* experimentation using position control is demonstrated. We see in Fig. 8 how the displacement follows a sinusoidal function and then after two cycles, the displacement follows a ramp function. This ramp function is necessary to statically calculate the stiffness of the scaffold in real time. If the stiffness was calculated during normal mechanostimulation, we are likely to include damping and



Fig. 6. Tensile testing of the robotic bioreactor compared to the IMADA tensile testing machine. The mean (bold line) and standard deviation (shaded area) of the three trials are plotted for Ecoflex 00-10 and 00-50. The bold line with no shaded region are the mean of three trials for each of the Ecoflex samples tested with the IMADA tensile testing machine. The red and orange line are results for the PGSM scaffold tensile test conducted by the IMADA tensile testing machine and the robotic bioreactor respectively.



Fig. 7. Example of a control signal for stiffness calculation. (Top) Displacement of the PGSM scaffold following a ramp signal controlled by the position controller. The controller displaces the pre-tensioned scaffold to 3.5mm at a steady rate of 0.7mm/sec and then stops. (Bottom) Force and displacement measurements from the bioreactor during the ramp displacement.



Fig. 8. Example of the robotic bioreactor's capabilities to enable mechanical stimulation of the tissue scaffold and derive its stiffness during its operation. (Top) The displacement of the rack following a sinusoidal waveform (blue) interspersed with ramp function at regular intervals. (Middle) Force measurements taken throughout experiment. (Bottom) A stiffness measurement is calculated at the same timestamp as when the ramp reaches its maximum displacement for 10 seconds.

elastic behaviour in the measurements without accounting for it mathematically.

## VII. IN VITRO PROTOCOL

The following protocol was designed for future experimentation to validate the bioreactor (Fig.9). PGSM scaffolds will be seeded with human dermal fibroblasts under aseptic conditions and cultured in a humidified incubator at 37°C, 5% CO2 to adhere to the scaffold for 24 hours. Once seeded, the scaffolds will be divided into three sets; one two-dimensional (2D) control, one 3D scaffold with no mechanical stimulation applied and one 3D scaffold with mechanical stimulation applied by the robotic bioreactor. To provide the cells with nutrition, 25 ml of medium will then added into the chamber. The medium will be prepared by combining Dulbecco's modified eagle's medium (DMEM) with Fetal Calf Serum, penisilin, fungizone and glutamine. The first set will be stimulated by the robotic bioreactor, the second set will be stimulated by Ebers [7] (as reference) and the last two scaffolds will be used as a 3D control. The 2D control will be proceeded by seeding the same number of cells onto tissue culture plastic (TCP) and will be placed in the same incubator. This 2D control will be used as the reference as the cells can be visually observed using a microscope.

For the case of the bioreactor introduced in this paper, the scaffolds used need to provide sufficient structural support for the cells [21], whilst maintaining elastic properties and resilience against wear [?][22] or plastic behaviour [23].

Sterility of the bioreactor is ensured through disinfecting all components and tools after every experiment. The ABS material of the chamber cannot be autoclaved since autoclave temperatures are higher than the ABS melting point[24]. Consequently, to disinfect the bioreactor, 70% isopropanol will be used since it is reported to disinfect more thoroughly in comparison with 70% ethanol [25].

The experiment will run over multiple days in the controlled environment; at body temperature (37°C), 5% CO<sub>2</sub>, 21% O<sub>2</sub> and 0% humitidy (to protect the electrical components within the bioreactor). Two different assays will be used to evaluate the performance of stimulation; resazurin, to acquire the metabolic rate of the cells [26], and picrosirius red assay, to determine collagen production [27].

Preliminary testing of the robotic bioreactor was conducted with a CSS mounted to the clamps and then placed inside an incubator along with four CSS in a petridish, one CSS in the Ebers bioreactor [7] (Fig.9). From this 3 days static experiment, it was verified that the chamber of the bioreactor was able to maintain a sterile environment from visual inspection of the cell media in comparison to the other controls. The viability of using the electronic components within an incubator was confirmed from this experiment as the components were still functional after 3 days.

### VIII. DISCUSSION AND CONCLUSIONS

In this paper, we introduce the concept of a novel robotic bioreactor equipped with the fundamental requirements to achieve stiffness-based force control in the future and validate these features with benchtop experiments. It is crucial to have a validation before moving forward with *in vitro* 



Fig. 9. Experimental set up to validate the chambers ability to maintain a sterile environment within an incubator for 3 days. Control CCS in petri dish (yellow), CCS in Ebers [7] (green) and CCS in the Robotic Bioreactor (pink).

experiments, as there are more variables that can affect the results in such experiments.

The robotic bioreactor is able to administer a variety of stimulation regimes, including those not achieved by commercially available bioreactors, such as an intermittent and incremental sinusoidal waveforms.

This 3D printed bioreactor can be adapted to be used for compression stimulation as opposed to tension stimulation, as demonstrated in this paper, with design changes to the chamber and clamps only. Limitations of 3D printing centre around poor dimensional accuracy when printing parts such as the worm gear. Future iterations of the bioreactor will include using a combination of 3D printed parts for elements of the bioreactor that is advantageous to change between experiments, such as the chamber and clamps and non-3D printed parts where accuracy is crucial, such as the worm gear and rack.

Implementing two different controllers into the bioreactor demonstrates its versatility as a tool. The position control appeared to be more robust than the force control as was less noise in the encoder readings than there was for the force sensor. The force control, however, is a novel approach to induce mechanostimulation onto a CSS. Both control methods can be used to achieve stiffness-based control which may be a step closer to achieving optimum mechanostimulation for CCS where the force applied to the an evolving scaffold is truly constant throughout an experiment.

The next steps include the validation of the mechanostimulation signals of the robotic bioreactor to regulate and optimise the cell-seeded scaffold *in vitro* in week-long experiments.

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APPENDIX B. PAPER 1

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