ADVANCED ELECTROMECHANICAL METHODS TO CULTURE ADULT MYOCARDIUM \textit{IN VITRO}

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Declarations

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Abstract

The adult heart is a terminally differentiated organ that responds to changes in its environment by phenotypic adaptations. This process involves electrical, mechanical, structural, and molecular changes and it is termed cardiac remodelling or plasticity. Understanding the mechanisms behind remodelling is crucial in understanding cardiac physiology and pathology and the transition between the two. A canonical driver of remodelling is mechanical load.

One method to examine plasticity in response to load is to subject cardiac tissue to different loads during in vitro culture and examine its temporal response and the terminal remodelled phenotype. There are multiple cardiac preparations used for in vitro culture, including engineered heart tissues, isolated cardiomyocytes, trabeculae, and papillary muscles. Throughout this thesis, we used a novel cardiac preparation known as living myocardial slices (LMS). These are 300 µm thick slices prepared from the explanted hearts of animals or humans. LMS are organotypic, meaning that they maintain the cellular stoichiometry, functional, structural, metabolic, and molecular profile of the tissue from which they are prepared. LMS were used as they offer several advantages compared to other models.

To date, mechanical load has been studied in vitro using either auxotonic or isometric loading protocols. These have enabled the progression of the field and our understanding of the effects of load on the myocardium. However, they are oversimplified as they fail to recapitulate the mechanical events of the in vivo cardiac cycle, which broadly include isometric contraction followed by ejection, isometric relaxation, and diastolic refilling.

In this thesis, we aimed to develop a culture platform to recreate the fine sequence of mechanical events that occur during the in vivo cardiac cycle and apply them in vitro on LMS. We did this by using a Three-Element Windkessel model to describe afterload and sarcomere length to describe preload. As both preload and afterload were parametrised in our platform, we were able to culture LMS under a range of pathophysiological loads.

In Chapter 3, we cultured LMS for 3-days under physiological load (normal preload, normal afterload), pressure-overload (high afterload, normal preload), or volume-overload (high preload, normal afterload). Our results show that LMS demonstrate distinct functional, structural, and molecular profiles with activation of both shared and unique gene networks as a function of mechanical load profile applied to them. To our knowledge, this was the first
time that cardiac tissue has been cultured and studied \textit{in vitro} under a mechanical load protocol that recreates the \textit{in vivo} cardiac cycle

In Chapter 4, we show the development of MyoLoop, a patented bioreactor designed to culture LMS \textit{in vitro} under the advanced electromechanical stimulation protocol developed in Chapter 3, which enables recreation of the \textit{in vivo} cardiac work loop. We describe the novelties, advantages, and limitations of MyoLoop relatively to other culture set-ups and commercially available systems.

Finally in Chapter 5, we shift our attention to acute physiological studies that examine transmural mechanical heterogeneity in the adult rat LV wall. To do that, we leverage the unique method of preparation of LMS, which involves the sequential generation of intact slices from the subendocardium to the subepicardium. We show that different layers of the left ventricular wall have different mechanical properties and provide a physiological conceptual framework for the presence of these.
Publications


Presentations


Exploring Mechanical Load-Induced Cardiac Remodelling Using a Novel Organotypic Myocardial Slice Model. **Oral Presentation** at Keystone Symposia Charting a New Course for Heart Failure, Colorado, USA 2020.

Exploring Mechanical Load-Induced Cardiac Remodelling Using a Novel Organotypic Myocardial Slice Model. **Poster Communication** at Biophysics Annual Meeting, San Diego, USA 2020.

Mechanical Heterogeneity Across the Left Ventricular Wall – A Study using Intact Multicellular Preparations. **Poster Communication** at ESC Conference; Paris, France 2019.


Physiological noradrenaline concentrations improve the functional and structural characteristics of cultured myocardial slices. **Poster Communication** at Experimental Models in Physiology; Exeter, UK 2018.
Patents

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For mom and dad
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List of Abbreviations

2EWK: 2-Element Windkessel
3EWK: 3-Element Windkessel
AOP: aortic pressure
APD: Action potential duration
BNP: brain natriuretic peptide
Ca: arterial compliance
CAD: Computer aided design
CCL: creative common license
CO: Cardiac output
DAQ: Data acquisition system
ECM: Extracellular matrix
EDPVR: End-diastolic pressure-volume relationship
EDV: end-diastolic volume
EDL: end-diastolic length
EDP: end-diastolic pressure
EHT: engineered heart tissue
ESPVR: End-systolic pressure-volume relationship
ESV: end-systolic volume
ESL: end-systolic length
GPIO: general purpose input output
GUI: general user interface
HF: Heart failure
hERG: human Ether-à-go-go-related gene
hiPSC-CMs: human-induced pluripotent stem cell-derived cardiomyocytes
I_{Na_l}: Late Na\(^+\) ion channel
IQR: Interquartile range
LMS: living myocardial slice
LV: left ventricle
LVP: left ventricular pressure
MMP: matrix metalloproteinase
mTOR1: mechanistic target of rapamycin 1
MYL2: Myosin light chain 2
PCB: printed circuit board
PI3K/Akt/mTOR: phosphoinositide-3 kinase/protein kinase B/mammalian target of rapamycin
PLA: polylactic acid
PVA: pressure-volume area
QMH: Queued message handler
Ra = arterial impedance
Rc = characteristic peripheral resistance
RL: Resting muscle length
ROS: Reactive oxygen species
RV: Right ventricle
SL: Sarcomere length
TAC: transthoracic aortic constriction
TdP: Torsade-de-Pointe
TTL: Transistor-transistor logic
VI: Virtual instrument
WGA: wheat germ agglutinin
Chapter One

Introduction
1.1 Cardiac remodelling

Cardiac remodelling or plasticity describes the molecular, cellular, and extracellular changes undergone by the heart in response to environmental stimuli, manifesting as changes in size, shape, geometry, and function of the heart (Azevedo et al., 2016). They occur in response to both physiological and pathological stressors (Naud, Guasch & Nattel, 2010) (see below). In this PhD thesis we aimed to develop an *in vitro* tissue platform to study cardiac remodelling. We begin by examining pathological and physiological remodelling and their relation to the pumping operation of the heart.

1.1.1 The core operation of the heart

The principal function of the heart is to maintain cardiac output (CO) to energetically demanding tissues. CO is governed by a set of fundamental equations related to heart rate (HR), stroke volume (SV), mean arterial pressure (MAP), and total peripheral resistance (TPR) (equations (1) & (2)).

\[
CO = SV \times HR \quad (1)
\]

\[
CO = \frac{MAP}{TPR} \quad (2)
\]

These equations can be used to relate the essential operation of the heart to a range of stressful conditions. For example, in response to fight-or-flight responses, an increase in CO is needed to take us away from danger. Activation of the adrenergic system increases HR and SV via \(\beta_1\)-adrenergic receptor, while \(\beta_2\)-adrenergic receptors decrease TPR, resulting in increased CO (Triposkiadis et al., 2009). Although these responses are evolutionary advantageous in the short-term, when these systems are chronically activated, they cause the heart to undergo maladaptive remodelling and promote cardiac dysfunction (Hill & Olson, 2008).

1.1.2 Physiological versus pathological remodelling

Not all remodelling is pathological; physiological remodelling is seen when heart size increases in response to exercise; this permits athletes to increase their max CO (Mihl, Dassen & Kuipers, 2008). Distinct types of physiological remodelling are seen with different types of training (e.g. strength or endurance) (Mihl, Dassen & Kuipers, 2008), and are largely driven by the different haemodynamic load experienced by the heart with each. For example, endurance training is characterised by long-intervals of high SV, HR, and moderate increases
in mean arterial pressure (MAP), which causes the CO to increase from 5 L/min at rest to ~40 L/min during peak activity (Ekblom & Hermansen, 1968). This represents a state of volume-overload and causes the ventricular dilation with expansion of both the heart’s internal diameter and thickness. (Mihl, Dassen & Kuipers, 2008)(Pluim et al., 2000). This type of physiological hypertrophy is known as eccentric hypertrophy and is also seen in pregnancy (Mesa et al., 1999). In contrast, strength training causes sudden and large increases in both systolic and diastolic blood pressure, with milder changes in CO, HR, and SV (Mihl, Dassen & Kuipers, 2008). This causes concentric cardiac hypertrophy with primarily increased wall thickness (Hill & Olson, 2008)(Mihl, Dassen & Kuipers, 2008). In contrast, when haemodynamic load is decreased, such as with space flight or prolonged bed rest, cardiac atrophy can occur (Perhonen et al., 2001). These examples highlight that the heart has massive potential for physiological remodelling, dependent upon the type of haemodynamic stimulus applied (Pitoulis & Terracciano, 2020).

With persistent stress or injury to the heart, the myocardium undergoes pathological remodelling. This is characterised by harmful phenotypic changes, such as fibrosis, inflammation, cell death and loss of contractility (Burchfield, Xie & Hill, 2013). The contemporary consensus holds that like in physiological remodelling, pathological changes are driven by activation of compensatory systems such as the renin-angiotensin aldosterone (RAAS) as well as the adrenergic axis, which aim to uphold CO in the short-term in the face of injury (Burchfield, Xie & Hill, 2013). Though overlap exists in the remodelling response (e.g. cardiac hypertrophy, proteolysis, etc.), pathological phenotypic changes are not overtly seen during physiological remodelling, at least initially (Pitoulis & Terracciano, 2020). For example, pathological hypertrophy is associated with activation of fetal genes, altered Ca²⁺ handling, fibrosis, metabolic reprogramming and mitochondrial dysfunction, whereas physiological hypertrophy is associated with signalling for cell survival, energy production, and angiogenesis at a rate equivalent to that of the increase in wall thickness (Nakamura & Sadoshima, 2018). In general, binary classification of remodelling may be too simplistic; rather it is more likely that remodelling occurs across a continuous spectrum (Dorn, 2007). It is likely that one of the differentiators is the intermittent nature of physiological stimuli (e.g. exercise) in contrast to the continuous, unresolved pathological stimuli (e.g. loss of contractility following myocardial infarction (MI)) (Carabello, 2014).
1.1.3 Mechanical load as a driver of remodelling

Perhaps the most important driver of cardiac remodelling is mechanical load – that is, the forces that act on and are acted upon by the heart (Pitoulis et al., 2019b). At minimum, an understanding of the load experienced by the heart at any given time requires quantification of preload and afterload.

Preload is the end-diastolic pressure (EDP) or volume (EDV) of the myocardium prior to electrical depolarisation and the onset of contraction. Fundamentally, preload maintains the steady-state of the closed-loop vascular system by linking venous return and CO. For example, increased venous return, increases diastolic stress (i.e. preload), increasing SV, and CO according to equation (1) and as shown in Figure 1.1A. Afterload is the load against which the heart needs to contract to eject blood into the vasculature during systole; it is largely determined by TPR (Figure 1.1B). For example, an increase in TPR decreases SV, and decreases CO according to equation (2) (Pitoulis & de Tombe, 2019). Not only afterload and preload but also the intrinsic inotropic state, also known as contractility, of the myocardium determines CO (Vincent, 2008) (Figure 1.1C). This is linked to circulating catecholamines, which sensitise the heart to contract more forcefully at a given preload, but also to the health/disease status of the heart.

Pathological increases in preload and afterload are referred to as volume- and pressure-overload, and are generally agreed to result in distinct cardiac phenotypes (Opie et al., 2006). In this PhD project, we developed an *in vitro* tissue platform based on the living myocardial slice (LMS) model (see 1.2.2) to a) recreate and subject LMS to volume- or pressure-overload, so as to b) study the remodelling response to mechanical overload *in vitro*. Prior to our discussion on the LMS model, the physiology and initial remodelling response to each overloaded profile is briefly discussed. For a detailed description of phenotypic outcomes in compensated and decompensate states please see (Pitoulis & Terracciano, 2020).
Figure 1.1: Pressure-volume loops with different preload, afterload, or inotropic state

A, B, C) Effect of changing preload (EDV) or afterload respectively on work loop morphology and SV. The main determinant of afterload is TPR. C) Increased inotropic state results in greater SV at the same preload and afterload. Reprinted by permission from [Springer Nature][Springer][Heart of the Matter: Cardiac contractility] by F.G. Pitoulis & de Tombe [CCC] (2019).
1.1.3.1 Pressure-overload

Perhaps the most prominent example of pressure-overload is hypertension, the single most important risk factor for development of heart failure (HF), with as much as 75 % of HF cases demonstrating antecedent hypertension (Burchfield, Xie & Hill, 2013)(Go et al., 2013). Other examples of pressure-overload include aortic stenosis (Carabello, 2013), aortic non-compliance (stiffening), and congenital conditions such as aortic coarctation (Groenemeijer et al., 2008).

In vivo, one of the most common ways to investigate pressure-overload induced remodelling is via thoracic aortic constriction (TAC) (Kuang et al., 2013). TAC increases afterload and for ejection of blood to occur left ventricular pressure (LVP) must rise to supraphysiological amounts (Figure 1.2). An increase in LVP increases myocardial wall stress, \( \sigma \), according to Laplace’s law of the heart (equation (3)) (Grossman, Jones & McLaurin, 1975). According to the seminal theory proposed by (Grossman, Jones & McLaurin, 1975), this is the principal driver of the hypertrophic response. Specifically, an increase in wall thickness, \( h \), with constant wall radius, \( r \), offsets the elevated LVP, normalising \( \sigma \), as shown by:

\[
\sigma = \frac{LVP \times r}{2h} \tag{3}
\]

This type of response is known as concentric hypertrophy (Schiattarella & Hill, 2015), and is generally considered to be an adaptive compensatory response to elevated afterload . At the cellular level, it is characterised by addition of sarcomeres in parallel (Schiattarella & Hill, 2015), which widen cardiomyocytes (Pitoulis & Terracciano, 2020).

The hypothesis that systolic load on its own is the driver of the hypertrophic response has been questioned. In a series of in vitro experiments, (Guterl et al., 2007) showed that culture of papillary muscles under isometric contractions with or without the contraction uncoupler BDM (i.e. decreased or normal systolic stress), led to the same type of cardiomyocyte hypertrophy. In contrast, papillary muscles which could shorten, did not show cardiomyocyte hypertrophy (Guterl et al., 2007). Thus, it has been suggested that rate and extent of muscle shortening may be more important than systolic stress for governing hypertrophy (Guterl et al., 2007). In particular, a decreased SV due to high afterload (Figure 1.1B) in pressure-overload may activate the hypertrophic response.

Whether hypertrophy is required and ultimately beneficial has also been put into question (Crozatier & Ventura-Clapier, 2015). Meerson argued that initial compensatory hypertrophy is followed by decompensation and HF (Meerson, 1969). The Framingham
Heart Study showed that an increase in LV mass was the most important risk factor for cardiovascular mortality (Manyari et al., 1990). Furthermore, (Hill et al., 2000) demonstrated that if the hypertrophic response is blunted in mice exposed to TAC, LV function is preserved. Preserved haemodynamic parameters with blockade of hypertrophy have also been reported by others (Choukroun et al., 1999)(Shimoyama et al., 1999). Collectively, such findings suggest that normalisation of stress through hypertrophic adaptations in the face of increased afterload may not be necessary for maintenance of contractile performance (Hill et al., 2000). Yet, others have shown that the hypertrophy is required and beneficial. For example, inhibition of stromal interaction molecule 1 (STIM1), a cardiomyocyte growth regulatory molecule, blunts hypertrophy but accelerates deterioration to HF (Schirone et al., 2017)(Hulot et al., 2011). Likewise, prevention of hypertrophy in mice models lead to increased mortality and cardiac dysfunction (Rogers et al., 1999)(Meguro et al., 1999). In view of such polarising findings, determining whether hypertrophy is ultimately beneficial may become difficult.

1.1.3.2 Volume-overload

Conditions of volume-overload are characterised by pathologically elevated preload, or EDV (Pitoulis & Terracciano, 2020). These are typically seen with valvular insufficiencies (e.g. mitral regurgitation, aortic regurgitation) (Carabello et al., 1989), aorto-venous shunts (Abassi et al., 2011), and anaemias (Metivier et al., 2000).

In response to volume-overload, the myocardium undergoes eccentric hypertrophy, characterised by increased internal diameter whereby cardiomyocytes lengthen with addition of sarcomeres in series. This can occur with or without corresponding changes in wall thickness (Urabe et al., 1992). If wall thickness does not change, the h/r ratio decreases according to equation (3), with pathologically high elevations in myocardial stress, promoting maladaptive remodelling and decompensation (Toischer et al., 2010)(Carabello et al., 1992). In contrast, if wall thickness increases, the h/r ratio remains constant, and adaptive hypertrophy occurs as seen in volume-overload in response to endurance in sport athletes (Hoogsteen et al., 2004)(Mihl, Dassen & Kuipers, 2008).

One of the accepted laws of cardiac physiology is that of Frank-Starling, which states force output, and by extension SV, increases with increases in diastolic stress (Allen & Kentish, 1985) (Figure 1.1A). However, force generation occurs across a narrow spectrum of sarcomere length (SL), typically between 1.9 and 2.4 μm (Rodriguez et al., 1992). During the initial stages of volume-overload, the myocardium is forced to operate on the upper-end of
SLs (Pitoulis & Terracciano, 2020). Accordingly, addition of SLs in series ‘pulls-back’ the
SL, maintaining the sensitivity of the force-SL relationship. This is demonstrated when
isolated neonatal cardiomyocytes are stretched along their longitudinal axis, causing an acute
increase in their SL, which however normalises back to original resting length after ~ 6 h
(Mansour et al., 2004). This process of sarcomere addition, which has been extensively
described elsewhere (Russell et al., 2010), seems to be regulated by protein kinase C-ε (PKC-
ε), focal adhesion kinases (FAKs), as well as intercalated disks (IDs) (Mansour et al.,
2004)(Yu & Russell, 2005). In this thesis (Chapter 3), we show that both the sensitivity and
the spectrum of operation of the force-SL relationship, that is the range at which the muscle is
responsive to stretch, are progressively lost with excessive diastolic preload, and hypothesize
that although this is an initially adaptive intrinsic myocardial mechanism, aimed at enabling
the heart to resume its pumping operation, it ultimately results in overt decompensation
(Figure 1.3)(Pitoulis et al., 2021).
Figure 1.2: Pressure-volume stress trajectories in response to pressure- or volume-overload

A) LVP at the onset of overload, or normal load. Normalisation of myocardial stress due $h/r$ compensatory changes. B) PV loops at the onset of pressure-overload, volume-overload, or normal load. ESPVR, EDPVR: end-systolic and end-diastolic pressure-volume loop relationships respectively. Figure adapted from (Pitoulis & Terracciano, 2020) under CCL.
The Frank-Starling law states that in response to increased EDV or length force output increases. In volume-overload prolonged elevations in diastolic stress cause the relationship to shift to the right, decreasing both the tissues force-length spectrum of operation and the sensitivity to stretch. This change in relationship is shown in Chapter 3.
1.1.3.3 Convergence and divergence in pressure- and volume-overload

Pressure- and volume-overload apply discrete mechanical load profiles to the working heart. However, the extent to which the remodelling responses are distinct phenomena or different events across a common spectrum is unclear. At least initially, each load profile seems to lead to distinct phenotypes, underlined by activation of unique signalling cascades (Miyazaki et al., 2006). For example, activation of Ca\(^{2+}\)/calmodulin dependent protein kinase (CAMKII), an intracellular serine/threonine kinase that mediates the hypertrophic response, has been shown in pressure- but not volume-overload (Backs et al., 2009)(Toischer et al., 2010). Likewise, collagen deposition is increased in pressure- (Bishop & Lindahl, 1999) but decreased in volume-overload (Hutchinson et al., 2011)(Wilson & Lucchesi, 2014). On the other hand, features of eccentric remodelling have been noted in patients with aortic stenosis, a state of pressure-overload (Villari et al., 1995) and similar changes in the isoform composition of titin, a sarcomeric protein, have been noted in pressure and volume-overload (Hutchinson et al., 2015)(You et al., 2018). Distinguishing the responses to pressure- or volume-overload may be even harder as many conditions, including myocardial infarction, expose the myocardium to features of both pressure- and volume-overload (Opie et al., 2006). Ultimately, understanding what drives remodelling in response haemodynamic load is not only scientifically interesting but also clinically important as it may permit tailoring of treatment to specific phenotypes.

1.1.3.4 Studying remodelling in response to mechanical load

Most contemporary cardiac research of mechanical load relies on experiments conducted in vivo. For the study of pressure- and volume-overload the commonest approaches include TAC and valvular insufficiency or aorto-venous shunt animal surgical models respectively (Corin et al., 1987)(Toischer et al., 2010). Although animal models are a fundamental tool in basic cardiovascular research, they can pose several challenges in studying load-induced remodelling.

The mechanical axis is continuously interacting with multiple other organ systems, to ensure adequate CO is maintained to the body. Even minor alterations in preload and afterload are met by activation of multiple neurohormonal pathways including the adrenergic axis, and RAAS. Given that both mechanical load and neurohormonal systems independently drive remodelling (You et al., 2018)(Triposkiadis et al., 2009), their simultaneous activation in vivo decreases our power to pinpoint cause and effect; this is particularly the case when one system is experimentally disturbed, which causes the other systems to be inadvertently disturbed.
disturbed as well (Russell et al., 2010). For example, although volume-overload is normally well-tolerated initially, if it occurs concurrently with blunting of the adrenergic axis, rapid and persistent cardiac deterioration occurs (Shah et al., 2000). Thus, studying the effects of the mechanical axis in isolation can be challenging in vivo.

A complementary strategy is the use of in vitro models, which provide a more reductionist approach to answer scientific questions that aim to study the mechanical axis alone in absence of other interacting variables (Habeler, Peschanski & Monville, 2009)(Pitoulis et al., 2019b).

1.2 In vitro models in cardiac research

Contemporary research follows a gradual progression from preclinical to clinical experiments. Preclinical studies utilise in vitro, ex vivo, and in vivo models and provide the data onto which most clinical studies are founded.

All wet experiments conducted in this thesis were on living myocardial slices (LMS), a novel in vitro cardiac model. In this section we first consider the properties of an ideal cardiac model. We then examine LMS and compare them with other models. We discuss current methods used to study remodelling and the protocols employed to simulate mechanical load on LMS in vitro. Finally, we examine current and potential future applications of the model.

1.2.1 The ideal cardiac model

There are many different cardiac research models, which can broadly be arranged across a complexity spectrum from subcellular and cellular systems to multicellular preparations such as papillary muscles, engineered heart tissues (EHTs) and LMS, to in vivo animal models (Figure 1.4).

Each model has unique advantages and limitations. In vitro models are particularly important as they form the testbed before animal experiments. The ideal in vitro model is one that maximises:

- Pathophysiological relevance
- Extrapolation of findings to human tissue
- Mechanistic insight
- Capability for high-throughput experiments

Pathophysiological relevance means that the physiological and pathological environment simulated in vitro is akin to that found in vivo. For example, to accurately simulate pressure-
or volume-overload both afterload and preload must be reproducible in vitro. Furthermore, and particularly for studying myocardial remodelling, key cardiac properties including cell-cell interactions, heterocellularity and ECM, must be adequately reflected in vitro as they can determine the response to remodelling stimuli (Perbellini et al., 2018a), and the final myocardial phenotype developed in pathology (Mathur et al., 2016).

Findings made in animals often do not extrapolate to humans (Kofron & Mende, 2017)(Fang & Casadevall, 2010)(Perry & Lawrence, 2017). Part of the explanation for this lies in differences in physiology between species (Liu & Antzelevitch, 1995) such as heart size and rate, ion channel densities, and molecular and functional signatures (Bassani & Bers, 1994)(Bers et al., 1993). For translation of findings, the ideal cardiac model must either reliably recreate human myocardial properties or permit the use of human tissue from disease or donor samples. In fact, much of modern basic cardiac research, and particularly within the field of maturing EHTs, has focused on the former, by creating development of substrates that aim to mimic the in vivo myocardium and its functional and structural properties(Hirt, Hansen & Eschenhagen, 2014).

Ideally, an in vitro model should allow for control of independent variable(s) and measurement of dependent variable(s) with little off-target or interacting effects. The extent to which a model can do this relates to its complexity (Pitoulis et al., 2019b). In general, it is easier to control the variable in question in vitro due to its reductionist nature. This permits greater degrees of causality and the uncovering of cell-specific effects. For instance, apelin is an endogenously synthesised inotropic peptide (Peyronnet et al., 2017); understanding its cardiomyocyte specific effects (e.g. contractility) is more challenging within a multicellular in vitro model as the off-target effects of apelin on other cell types (e.g. endothelial cells)(Masoud et al., 2020) may counteract or accentuate cardiomyocyte specific effects (Farkasfalvi et al., 2007). Overall, the more complex a model, the closer it is to the in situ environment, and thus the greater the probabilities that results will extrapolate to in vivo models and subsequently to the clinics. On the contrary, the more reductionist the model, the less the degrees of freedom, and thus the more likely that cause and effect relationships will be robustly established (Figure 1.4).

Finally, the ideal in vitro model should allow for high-throughput experiments. In such systems parallel and/or combinatorial interrogation of multiple different questions is possible, accelerating therapeutic discoveries. This is particularly important for experiments conducted on scarcely available tissue, such as human hearts, and more so if these are long-term culture experiments. Currently, there are two main obstacles in establishing high-throughput systems
that can uphold the other principles of an ideal cardiac preparation. First, for
pathophysiological relevance to be maintained, complex bioreactors are needed that are able
to simulate the *in vivo* electromechanical behaviour of the heart at the *in vitro* level, both in
health and disease (Tandon *et al.*, 2009a)(Vunjak Novakovic, Eschenhagen & Mummery,
2014). This is not easy, as the heart is the largest bioelectric source in the body and its
mechanical operation is complex, dynamic, and under continuous negative feedback
regulation (Tandon *et al.*, 2009a)(Pitoulis *et al.*, 2021). Secondly, for multiple parallel
experiments the *in vitro* model in question must allow for multiple samples per specimen to
be collected simultaneously.
Figure 1.4: Tornado plot of cardiac research model complexity
Simplified image showing cardiac research models arranged across a complexity spectrum. Each model has unique advantages and limitations. LMS are intermediate complexity in vitro models.
1.2.2 Living myocardial slices (LMS)

LMS, also known as cardiac slices, are 300 μm thick living cardiac preparations of intermediate complexity (Figure 1.5). They are prepared from the hearts of small and large mammals, including human donor and biopsies, using a high-precision vibrating microtome (Brandenburger et al., 2012)(Watson et al., 2017)(Perbellini et al., 2018b). They are described as organotypic as they have the functional (e.g. contractility, electrophysiology), structural (extracellular matrix (ECM), cell-cell interactions, and stoichiometry), and molecular properties of the tissue from which they are obtained.

They were first used in 1946 to study the effects of changes in circulating volume due to haemorrhagic shock on rat metabolism, and at the time were prepared using hand-held blades (Burdette & Wilhelmi, 1946)(Barron, Sights & Wilder, 1953)(Pearson, Hastings & Bunting, 2017). Since then, the model has seen tremendous growth (Figure 1.6). This can be partly attributed to the development of high precision vibratomes, which can automatically section heart samples into LMS with minimal Z-axis error, and thus tissue injury (Pitoulis et al., 2019b). Together with recurrent LMS preparation protocol refinements, described extensively in (Watson et al., 2017) and 2.4 in this thesis, the model has seen rapid adoption highlighting its potential for cardiovascular research and widening its capabilities (Pitoulis et al., 2019b). Although LMS can be used for a range of experiments one research area that has gained more traction are long-term in vitro culture experiments (Fischer et al., 2019)(Watson et al., 2019)(Pitoulis et al., 2021). With such experiments the temporal and long-term effect of interventions on the myocardium can be investigated. Different labs, including ours, have developed different culture methods for long-term in vitro studies; these are discussed in 1.2.4.
Figure 1.5: Living myocardial slices (LMS)

A) Cartoon schematic of LMS. LMS are 300 μm thick organotypic preparations. They can be prepared from multiple species including humans. The typical LMS size is 8 x 10 mm in width and length respectively. B) Picture of LMS. A heart sample or biopsy is cut into 300 μm sections using a vibratome, yielding an LMS (B, left). The LMS is then trimmed to the required size and shape for use in subsequent experiments. Figure adapted from (Pitoulis et al., 2019b) under CCL.
Figure 1.6: LMS model uptake within scientific research

Papers published between 1943 and 1949 that use LMS. A total of 181 papers were identified in the literature. Figure adapted from (Pitoulis et al., 2019b) under CCL.
1.2.3 Fitting LMS within the contemporary cardiac research landscape

The method of preparation of LMS is mechanical, using a vibratome (Watson et al., 2017)(Brandenburger et al., 2012)(Kang et al., 2016) (see Chapter 2). In contrast to other potentially damaging protocols of in vitro model preparation (e.g. enzymatic digestion in cardiomyocyte isolation), there is preservation of the ECM and minimal cell injury (Watson et al., 2017)(Pitoulis et al., 2019b). In fact, only ~ 3% of all cells are damaged in an LMS, ensuring reliable functionality with multiple independent studies validating the preparation for its robust contractile (Watson et al., 2019)(Fischer et al., 2019)(Perbellini et al., 2018b)(Pitoulis et al., 2021), electrophysiological (Qiao et al., 2019)(He et al., 2019)(Dries et al., 2021), and metabolic performance (Watson et al., 2019)(Camelliti et al., 2011), as well as the underlying molecular signatures (Fischer et al., 2019)(Pitoulis et al., 2021)(Pitoulis et al., 2020)(Watson et al., 2019) (Figure 1.7).

Depending on the species used, multiple LMS can be prepared from single LV specimen, with up to 30 LMS per human 1 x 1 x 1 cm LV biopsy. Other tissue models, such as cardiac wedges, papillary muscles, and whole hearts have significantly lower specimen to sample ratio, with typically one wedge and two papillary muscles obtainable per heart (Pitoulis et al., 2020)(Watson et al., 2019). This combined with the need for expensive set-ups required to perform experiments, can significantly hamper model scalability and the potential for high-throughput experiments. For example, cardiac wedges require ex vivo perfusion systems (Di Diego et al., 2013), whereas whole hearts require complicated perfusion systems (Schechter et al., 2014). Although experimental set-ups (e.g. optical mapping, organ baths) are generally equally expensive for LMS as for other models, the higher number of samples obtainable in the former allows for parallel studies even when using scarce tissue (e.g. human HF tissue) (Pitoulis et al., 2019b).

Physiology-based studies can be conducted on freshly prepared LMS (Pitoulis et al., 2020). However, the field is rapidly moving towards the use of the model as a culture platform for the study of remodelling in response to long-term interventions. Of note is that culture of adult cardiac tissue and cardiomyocytes is notoriously difficult (see 1.2.4). When single cells or tissue are cultured in vitro, the artificial environment which typically lacks the in situ cardiac micro- and macro-environment, (Hill & Olson, 2008)(Schneider-Warme, Johnston & Kohl, 2018) leads to artificial changes in the model’s archetypical structure and function (Louch, Sheehan & Wolska, 2011)(Vunjak Novakovic, Eschenhagen & Mummery, 2014)(Zhang et al., 2010). As such, although isolated cardiomyocytes have been the
fundamental building blocks of modern cardiac research (Bazan et al., 2012) they can only be studied in a physiologically relevant context within short timepoints from preparation (Vunjak-Novakovic, Eschenhagen & Mummery, 2014). In contrast, LMS preserve the structure of the in situ myocardium, therefore ensuring the presence of micro- and macro-environmental cues (Watson, Terracciano & Perbellini, 2019). Other cardiac models such as EHTs do not yet fully replicate the in vivo cardiac architecture and cell ratios (Mannhardt et al., 2016), and though the hopes for these technologies in personalised medicine, disease modelling, and pharmacological testing are high, without robust model validation and interrogation to ensure a sufficiently matured adult-like phenotype, interpretation of results will remain challenging (Hirt, Hansen & Eschenhagen, 2014)(Hirt et al., 2014)(Ronaldson-Bouchard et al., 2018) (Table 0.1). Similarly, tissue models such as papillary muscles, wedges, and whole hearts are typically only studied for a few hours when placed on a rig, before undergoing rapid run down (Kang et al., 2016)(Watson et al., 2017). Although papillary muscles have been cultured in vitro using specialised muscle chambers (Janssen et al., 1998)(Guterl et al., 2007) the thickness of the model can impair O₂ diffusion to the core of the preparation (Guterl et al., 2007)(Watson, Terracciano & Perbellini, 2019)(Watson et al., 2017), confounding results. In contrast, LMS are 300 μm thick and as the O₂ diffusion barrier is 200 μm (Carmeliet & Jain, 2000) it can diffuse from both sides reaching the tissue core.

1.2.3.1 Limitations of the LMS model

LMS preparation involves cutting ventricular tissue from endocardium to epicardium in 300 μm sections. As differences may exist transmurally, it is important to consider the region of origin of each LMS or account for it by randomising LMS from different layers of the LV wall across the interventions examined, when conducting experiments. However, transmurality may be advantageous for novel research into physiology and the operation of the heart in vivo. Transmural differences exist in electromechanical, structural, and molecular properties of the heart as has been shown using permeabilised cardiac models or isolated cells (Cazorla et al., 2005)(Haynes et al., 2014)(van der Velden et al., 2011). LMS offer a novel approach to this problem by considering a model that is intact and functional. In Chapter 5, we leverage this unique aspect of LMS preparation to show that the mechanical properties of LMS from different layers of the LV wall are different, and that this may be an intrinsic mechanism to facilitate effective pumping operation of the heart in vivo (Pitoulis et al., 2020)(Dries et al., 2021).
Another point of consideration is LMS geometry and size. Although their size (~ 10 × 8 × 0.3 mm) permits physical manipulation of the model (e.g. stretch) without the need for complex set-ups (e.g. atomic force microscopy (Borin et al., 2018)(Teng, Loukin & Kung, 2014), or magnetic tweezers (Chen et al., 2015)), when LMS contract the stress and stretch vectors occur across a 2D plane. This contrasts with the 3D pressure generation and changes in volume in vivo. Although geometrical models can convert 2D to 3D vectors (De Tombe & Little, 1994)(Pitoulis et al., 2021), extrapolations to 3D relationships should be made with caution. Additionally, ventricular conformational changes in vivo include longitudinal, and circumferential shortening in sync with radial thickening. These changes are the result of 1) helical arrangement of cardiomyocytes across the LV wall, and 2) 5-10 cardiomyocyte sized microstructures, known as sheetlets (Nielles-Vallespin et al., 2017). Reorientation of sheetlets from low (diastole) to high (systole) angle during contraction has been suggested to underpin ventricular radial thickening and impaired sheetlet reorientation has been observed in dilated (DCM), and hypertrophic cardiomyopathy (HCM) (Nielles-Vallespin et al., 2017). The method of LMS preparation involves untwisting of the helical cardiomyocyte arrangement from epicardium to endocardium (given 300 μm model thickness), and by extension loss of physiological sheetlet structure. As such, conformational changes relating to radial thickening are not captured by the LMS model.

LMS are also suboptimal for studies of vascular biology. Like papillary muscles, trabeculae, and most EHT models, LMS rely on diffusion for O\textsubscript{2} and metabolic substrate supplementation. As such, flow across capillaries and vessels is minimal and their effects on the tissue dismal. The same principle applies to the study of hormonal, neuronal, or inflammatory influences on the model. As aforementioned advantages and limitations exist to the isolation of a system from the rest of the body, but ultimately care must be taken to avoid ‘over-translation’ of findings beyond what an in vitro preparation can afford.

Much like every other cardiac preparation, LMS have unique advantages but also limitations; LMS are not the ideal cardiac model, and currently, the latter is merely a theoretical construct. Ultimately, the choice of the appropriate experimental model depends on the scientific question being asked.
Figure 1.7: LMS structure, function, and molecular signatures

A) Canine LMS visualised in x20 magnification confocal microscopy stained with caveolin3 (cav3, red colour), and isolectin (white colour). B) LMS can be used for metabolic studies. Panel shows ATP to ADP ratio of freshly prepared human LMS using high performance liquid chromatography. C) T-tubule network of LMS after 24 hr culture at different preloads (sarcomere lengths ranging from 1.8- to 2.4 μm). D) Functional assessment of LMS. Panel demonstrates twitch force of human HF LMS paced at 0.5 Hz. Figure adapted from (Pitoulis et al., 2019b) under CCL.
Table 0.1: Comparison of *in vitro* and *ex vivo* cardiac models for cardiovascular research

<table>
<thead>
<tr>
<th>Feature</th>
<th>Isolated Myocytes</th>
<th>Papillary muscles</th>
<th>Whole Hearts</th>
<th>EHTs</th>
<th>LMS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>in vivo</em> proximity</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Throughput</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Degrees of causality</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Cost</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Capacity for long-term experiments</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Personalised assays</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
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</table>

**Note:** there is no ideal cardiac model. Each preparation has unique strengths and weaknesses, and choice of model depends on experimental question.
Cardiac tissue culture is a unique method to study cardiac plasticity and the long-term effects of pathological and therapeutic interventions on the myocardium. However, an *in vitro* system is by definition artificial, and it is therefore essential that cause-effect relationships due to the intervention in question are distinguishable from the biological noise induced by the artificiality of the *in vitro* environment (Miller, 1995). The main challenge in doing this reliably is the utter sophistication of the heart as an organ. Lack of cardiac micro- and macro-environmental cues, mechanical and electrical stimulation, and native tissue architecture as is seen *in vivo* contribute to this artificiality and can hinder translation of findings (Mitcheson, Hancox & Levi, 1998)(Louch, Sheehan & Wolska, 2011)(Zhang *et al.*, 2010). To address this, modern cardiac culture approaches attempt to recreate features of the *in vivo* environment *in vitro* by including mechanical and electrical stimulation, hormonal agents (Yang *et al.*, 2014b)(Ronaldson-Bouchard *et al.*, 2018)(Tandon *et al.*, 2009b), and cardiac tissue substrate cues (Jawad *et al.*, 2008) These approaches have collectively been termed biomimetic (Fischer *et al.*, 2019)(Watson *et al.*, 2019)(Miller *et al.*, 2020).

### 1.2.4.1 Simulating mechanical load during *in vitro* LMS culture

One of the earliest approaches to culture LMS was by (Brandenburger *et al.*, 2012), who kept human adult LMS in semi-porous air-liquid interfaces for 28 days. Although LMS were viable and responsive to pharmacological stimulation (e.g. β-adrenergic agents) at end-point culture, they had undergone significant culture-induced remodelling losing the archetypical functional (contractility, electrophysiology) and structural (increased tissue elasticity, loss of rod-shaped cardiomyocyte, downregulation of sarcomeric proteins) properties of adult cardiac tissue (Brandenburger *et al.*, 2012).

For a long time, the air-liquid interface was considered the gold-standard to culture LMS. However, many of the culture changes (e.g. no contractility, reorganisation of sarcomeres) effectively described an atrophic and minimally viable myocardium (Hill & Olson, 2008). At the same time as these observations were made, the field of regenerative medicine and EHT maturation was highlighting the importance of mechanical load for functional and structural maturation during culture (Tulloch *et al.*, 2011)(Zimmermann, 2013)(Jackman, Carlson & Bursac, 2016)(Hirt, Hansen & Eschenhagen, 2014)(Feric & Radisic, 2016). The natural course was assimilation of novel EHT methods into the culture of LMS, leading to an outflow of publications from our (Perbellini *et al.*, 2018b)(Watson *et al.*, 2019).
2019) and other laboratories (Miller et al., 2020) (Fischer et al., 2019)(Qiao et al., 2019) (Ou et al., 2019), which used mechanical load in different forms to culture LMS.

At the beginning of this PhD thesis, the two main methods to apply mechanical load on cardiac tissue in vitro, and on which all culture protocols were based were a) isometric, or b) auxotonic mode of contraction (Figure 1.8). These methods were published almost simultaneously by our lab (Watson et al., 2019) and (Fischer et al., 2019) respectively, and are examined in detail here and 2.13 Chapter Three 3.

In the isometric platform developed by (Watson et al., 2019), LMS are mounted on the posts of custom-made stainless steel stretchers, which are then immersed in biocompatible chambers (Figure 1.9). The chambers are placed inside an incubator, a peristaltic pump is used to recirculate media, and electrodes connected to a stimulator are immersed in the media inside the chamber providing field stimulation to the LMS (Figure 1.9). Up to four stretchers can fit within one chamber enabling a mid-throughput system. A hex screw controls the distance between the posts onto which LMS are mounted, such that tightening/untightening the screw allows for control of diastolic length of LMS. As muscle length and SL have a linear relationship, LMS can be stretched to the desired SL, and thus preload, by using a set of callipers and muscle length as a SL surrogate (Cleworth & Edman, 1969). Using this platform, (Watson et al., 2019) showed that when rat LMS are cultured at a preload of 2.2 μm SL for 24 hr and 1 Hz stimulation frequency their contractile, electrophysiological, metabolic, structural, and molecular phenotype is preserved compared to when they are cultured unloaded (1.8 μm SL) or overloaded (2.4 μm SL). Though 24 hr culture is a relatively short timeframe for culture studies, the protocol to load LMS was a significant step forward after the liquid air interface, and (Watson et al., 2019) showed that rabbit LMS can be cultured for 5 days at SL of 2.2 μm without a drop in baseline contractility.

A similar culture set-up was developed almost simultaneously by (Fischer et al., 2019). In this, LMS are mounted on a rigid post on one side and a flexible stainless-steel spring on the opposite side (Figure 1.9). When the tissue is stimulated to contract, the flexible post bends, and the LMS contracts auxotonically; that is force and tissue length are both changing simultaneously, in a combination of isometric and isotonic contraction (Keefe et al., 2010). One breakthrough of this system was the use of a magnetic analog system to measure the displacement of the flexible post. Given the posts stiffness and the displacement, the force generated by the LMS can be calculated and the LMS contractile behaviour monitored throughout culture. This is in contrast to the set-up by (Watson et al., 2019), which does not allow for monitoring of the LMS contraction; LMS are mounted on the stretchers and placed
inside the culture chamber, the experimenter has no feedback from the tissue; Furthermore, 
the system by (Fischer et al., 2019) was innovative in that it developed custom electrical 
stimulation and media agitation peripheral modules. Thus, except for an incubator, third-party
components are not required for conduction of experiments. Fischer et al., were able to
culture and maintain human adult LMS viable and contractile for up to 4-months using their
system (Fischer et al., 2019). Although this was a big step forward in the field, the LMS
showed significant culture-induced remodelling at end-point culture as demonstrated by the
diverging Frank-Starling arms and molecular signatures (Fischer et al., 2019). Additionally,
the LMS cultured by (Fischer et al., 2019) were paced at 0.2 Hz, and demonstrated an early
abnormal drop in contractility that was sustained for multiple weeks.

The main difference between the (Watson et al., 2019) and (Fischer et al., 2019)
platforms is the LMS mode of contraction. In the former, contraction is isometric with the
LMS beating against inflexible stainless-steel posts and effectively infinite afterload. As
afterload is constant and infinite, force development is a function of preload alone, according
to equation (4).

\[ \text{Twitch force} \propto f(\text{Sarcomere Length}) \quad (4) \]

In the (Fischer et al., 2019) platform, LMS are beating auxotonically against a flexible
post with tissue shortening and force development following a linear relationship (the
stronger the tissue the greater the shortening), according to equation (5).

\[ \text{Twitch force} \propto f(\text{Shortening velocity, Sarcomere Length}) \quad (5) \]

Both electromechanical protocols have been referred to as ‘biomimetic’, yet neither
accurately recreates the mechanical operation of the heart. In particular, both approaches fail
to capture the dynamic complexity of in vivo electromechanical events, evident by pressure-
volume trajectories (Figure 1.8). The in vivo cardiac cycle consists of sequential phases of
isometric/isovolumetric and isotonic/ejection contraction followed by isometric/isovolumic
relaxation and diastolic refilling. Purely isometric or auxotonic systems only partially capture
the in vivo pumping operation of the heart.

In this PhD, we aimed to develop a culture system that can simulate in vivo pressure-
volume loop trajectories at the in vitro level as LMS force-length loops with variable and
controlled afterload and preload. This would enable us to recreate physiological and
pathological mechanical load profiles to study remodelling. To do that, we utilised a
circulatory mathematical model known as Three Element Windkessel (3EWK).
Figure 1.8: Computer simulations of auxotonic, isometric, and \textit{in vivo} force-length loop trajectories
A) Auxotonic mode of contraction of LMS beating in (Fischer \textit{et al}., 2019) system. B) Isometric force-length trajectory of LMS beating in (Watson \textit{et al}., 2019) system. C) Force-length loops of the working heart \textit{in vivo}, consisting of distinct phases of isometric and isotonic contraction and relaxation. Figure adapted from (Pitoulis \textit{et al}., 2019b) under CCL.
Figure 1.9: Current laboratory systems for in vitro LMS culture
A, C) Culture platform developed by Fischer et al., 2019. LMS are loaded on flexible posts which allow auxotonic contractions. Measurement of the displacement of the flexible post allows quantification of the force generated by the tissue and monitoring of contractility during culture. The set-up consists of custom electrodes, and media recirculation/agitation system. Temperature is controlled using an incubator. B, D) Culture platform developed by Watson et al., 2019. LMS are mounted on inflexible stainless-steel stretchers, which allow adjustment of preload (diastolic length), while afterload is infinite and constant. The stretchers are placed inside the culture chamber, and the LMS paced using carbon electrodes connected to an external third-party stimulator. Media is recirculated by a peristaltic pump and the whole set-up is maintained at 37 °C using an incubator. Figure adapted from (Pitoulis et al., 2019b) under CCL.
1.2.4.2 Three Element Windkessel Model

Windkessel, which means ‘air chamber’ in German, is a model to describe the arterial blood pressure waveform within a circulatory system consisting of compliant elastic arteries and resistance vessels. The first description was proposed by Otto Frank, the prominent German physiologist, who postulated that blood ejection into the circulation is like a piston pumping water into an air-filled chamber (Frank, 1899). When the chamber fills with water, the air gets compressed, which subsequently causes the water to flow out of it. In the analogy, the air-filled chamber represents the compliant large vessels (aorta) which store mechanical energy during systole and release it in diastole (Figure 1.10).

Windkessel descriptions of the circulatory system are lumped models – that is, the physical properties of the entire vascular system are represented by parameters with physiological relevance and the pressure-flow relations at the entrance of the circulation (Westerhof, Lankhaar & Westerhof, 2009). They are typically solved by approximating the entire vascular tree as electrical circuit segments and then using empirical and mass conservation equations such as Ohm’s and Kirchhoff’s respectively. As such, blood flow and pressure are simulated by current, $I(t)$, and electrical potential, $V(t)$, whilst vascular resistance is simulated by resistors, vessel compliance by capacitors, and inertia of blood by inductors (Peter, Noury & Cerny, 2018). Two, three-, and four-element models exist with 2, 3, and 4 physiological parameters determining the system respectively.

In the first model proposed by Frank, a two-element Windkessel (2EWK) describes the cardiovascular system using a single resistor ($R$) and capacitor ($C$) (Figure 1.10). The former accounts for the total peripheral resistance of the entire arterial tree, whilst the latter accounts for the compliance of the aorta. The theoretical equation describing such a system is shown in equation (6). During diastole, blood flow or $I(t) = 0$ and the 2EWK reliably predicts that blood pressure in the aorta decays exponentially with a time constant determined by $R$ and $C$ (equation (7)). However, with the advent of electromagnetic flow and pressure-flow measurements it quickly became apparent that 2EWK systolic predictions were less vigorous (Westerhof, Lankhaar & Westerhof, 2009). A second resistor to account for the impedance of the proximal aorta was added to the 2EWK, yielding the 3EWK model. This consisted of arterial impedance ($R_a$), aortic compliance ($C_a$), and peripheral resistance ($R_c$). The theoretical model is described in equation (8).

$$I(t) = \frac{P(t)}{R} + C \frac{dP(t)}{dt} \quad (6)$$
Windkessel models are often used to simulate aortic blood pressure waveforms and the effect of compliant and resistive elements on these. For example, systolic blood flow at the aortic outflow trunk can be simulated using a time-varying sinusoidal waveform, with amplitude \( I_o \) and \( I(t) = 0 \) during diastole when the aortic valves are closed. If the differential equations are solved for electrical potential, \( V(t) \), the time-varying aortic blood pressure waveform is obtained, with different haemodynamic outcomes for different values of \( R_a, C_a \), and \( R_c \). The values of these can be estimated \textit{in vivo} using pressure-flow data. \( R_c \) can be calculated by dividing mean flow with mean pressure (Stergiopulos, Westerhof & Westerhof, 1999), \( R_a \) via pressure and flow Fourier transforms (Deswysen, Charlier & Gevers, 1980), and \( C_a \) by using the pulse pressure method (Stergiopulos, Meister & Westerhof, 1994). \textit{In vivo} these vary in health between 2-7 for \( R_a \), 0.1-0.6 for \( R_c \), and 0.1-0.3 for \( C_a \) in the dog (Stergiopulos, Westerhof & Westerhof, 1999). In conditions where afterload is increased (e.g. hypertension) \( R_a \) and \( R_c \) increase while \( C_a \) decreases in magnitude (Segers, Stergiopulos & Westerhof, 2000).

In this PhD thesis, we used the 3EWK mathematical model to mechanically load LMS (Chapter 3 and 5) during \textit{in vitro} culture. Within the \textit{in vitro} set-up, LMS generate force after being field stimulated to contract using electrodes. The 3EWK can then be solved for \( I(t) \) after converting force to pressure, or electrical potential, \( V(t) \), using Laplace’s law of the heart (equation (3)). Blood flow, \( I(t) \), is equal to rate of change of left ventricular (LV) volume and can be converted to muscle length using geometrical models (see Chapter 3 and 4). A length waveform is obtained in this manner for a particular pressure waveform (i.e. LMS force transient), which it then applied on the LMS in sync with the electrical field stimulation allowing us to control LMS rate and amplitude of shortening. As this can be done for any combination of \( R_a, C_a, \) and \( R_c \), a spectrum of mechanical loading conditions can be investigated. For example, when \( R_a \) and \( R_c \) are high, but \( C_a \) is low, mechanical overload is approximated as seen in aortic stenosis and aortic non-compliance. In contrast, when \( R_a \) and \( R_c \) are low and \( C_a \) is high, resistance is low while compliance is high, yielding conditions of mechanical unloading.
Figure 1.10: Two- and three-element Windkessel models
A) In the original Windkessel analogy a piston pumps water into a compliant air-filled chamber, which subsequently pushes the water out due to compression of the air. This mechanism was used in the past in firehoses. B) Two-element Windkessel model consisting of a capacitor and a resistor. C) Three-element Windkessel model consisting of two resistors and one capacitor; this model was used to mechanically load LMS in this PhD project.
1.3 LMS for basic and translational cardiovascular research

Prolonged culture of adult cardiac tissue in vitro opens frontiers for scrutinization of both basic science and translational research questions. In the following section we discuss a few applications of these.

1.3.1 Pathological and reverse remodelling

In the past decades, most cardiac therapies have centred around the use of agents that modify neurohormonal pathways, which when activated for extended periods of time cause detrimental deterioration to the function of the heart (Gheorghiade, De Luca & Bonow, 2005). Although such modalities have improved mortality and morbidity rates (Luscher, 2015), agents like angiotensin converting enzyme inhibitors, neprilysin inhibitors, mineralocorticoid receptor inhibitors, hydralazine, and nitrates all primarily act on the periphery. As such, it has been argued that we may be reaching a glass ceiling and development of novel agents that directly target the diseased myocardium are needed (Gheorghiade et al., 2016). Long-term culture of adult LMS could mediate that. For example, LMS could be subjected to mechanical overload and the pathological remodelling response examined (Pitoulis et al., 2021); identified pathways mediating the pathological response could then be blocked. Equally, diseased tissue from human or animal HF samples could be subjected to mechanical unloading, and therapeutic mediators of reverse remodelling pinpointed (Ibrahim et al., 2010); these could then be boosted by development of novel compounds (Pitoulis et al., 2019b).

1.3.2 Cardiac pharmacology

In developing novel therapeutic agents, the main current challenges revolve around identification of agent’s potential for cardiotoxicity, and lack of translation from in vivo animal models to humans.

Cardiotoxicity remains one of the foremost reasons for drug discontinuation (Nguyen et al., 2017). A common example is drug-induced Torsade-de-Pointe (TdP). This is a life-threatening tachyarrhythmia mechanistically linked to inhibition of the hERG channel (K$^+$ channel), and subsequent delayed ventricular repolarisation leading to prolongation of the ECG QT-interval (Shepard, Canavier & Levitan, 2007)(Anwar-Mohamed et al., 2014). In vitro cardiotoxic assays therefore often test therapeutic compounds against recombinant hERG channels (Hancox et al., 2008) using rapid screening modalities. Although this allows for identification of drugs that can block the channel, hERG inhibition does not guarantee clinical arrhythmia (Lu et al., 2008)(Li et al., 2016)(Yang et al., 2014a). This is because
Arrhythmias are not isolated phenomena but instead linked to tissue multicellularity, cell-cell coupling, and ultimately current sink-source mechanisms (Xie et al., 2010)(Bussek et al., 2012). The organotypic nature of LMS could make them an ideal preclinical testbed for drug screening, and studies have shown that when adult human HF LMS are exposed to QT-interval prolonging drugs in vitro, their field potential duration, a surrogate of the in vivo ECG QT-interval, is lengthened (Camelliti et al., 2011), validating their potential.

Long-term consequences of the drug in question can also be assessed with LMS culture. This is particularly important because a) cardiotoxic effects may require time to manifest, and b) acute and chronic drug effects may differ. Doxorubicin, a known cardiotoxic anticancer drug, has been cultured for 24 hr with adult rat LMS (Parrish, Gandolfi & Brendel, 1995)(Parrish et al., 1994). Signs of cardiac damage were demonstrated by decreased production of ATP and protein as well as increased production of injury markers such as creatinine kinase (Parrish, Gandolfi & Brendel, 1995)(Parrish et al., 1994). Dofetilide, a class III antiarrhythmic agent (K\(^+\) channel, I\(_{Kr}\), blocker) was until recently thought to only have no other electrophysiological effect (Yang et al., 2014a). In isolated adult mouse cardiomyocytes lacking I\(_{Kr}\), acute exposure to dofetilide does not change action potential duration (APD) or ion currents (Yang et al., 2014a); yet prolonged exposure (2 to 48 h) led to arrhythmogenic afterdepolarisations (Yang et al., 2014a). These effects were attributed to activation of the late Na\(^+\) channel (I\(_{Na-L}\)) after chronic drug exposure (Yang et al., 2014a). In similar fashion, when human HF LMS are acutely exposed to phenylephrine APD increases, yet chronic exposure leads to APD abbreviation (Kang et al., 2016). Uncovering of such effects and their consequences requires platforms that can maintain tissue viable and functionally relevant for prolonged periods of time.

Finally, LMS could be used in sync with other models for preclinical drug testing of potential therapeutic agents. Given that multiple LMS can be obtained from relatively small-sized human biopsies (1 x 1 x 1 cm), different drugs and/or concentrations could be examined simultaneously and chronically on LMS; this would allow for investigation of myocardial remodelling in response to the therapeutic intervention in question. As human tissue phenotype can vary based on patient status, with sufficient institutional organisation, specimens from different origin (e.g. HF patients, myectomies, or healthy donors) could be classified into discrete groups. The response of each patient group to each drug could then be scrutinised, homogenising patient populations, decreasing specimen variability, and identifying subpopulations that are most likely to benefit from specific drugs (Pitoulis et al., 2019b).
1.4 Aims and objectives of the project

This thesis addresses two hypotheses. First, that application of electromechanical stimulation in the form of force-length loops can enable the study of load-induced remodelling in vitro (Aim 1 & 2). Second, that intrinsic transmural differences exist across the LV wall and that these can be studied using LMS (Aim 3).

Aim 1
Develop a method to culture LMS using physiologically relevant mechanical preload and afterload for the study of load-induced remodelling in vitro (Chapter 3).

- To recreate the in vivo cardiac cycle in vitro.
  - To apply the 3EWK mathematical model on LMS in vitro.
- To culture LMS using mechanical load profiles that simulate physiological load, pressure-, and volume-overload.
  - To use this approach to study the effects of chronic mechanical overload on the myocardial phenotype.

Aim 2
Develop a LMS culture bioreactor to increase the accessibility of the culture methodology developed in Chapter 3 (Chapter 4)

- To develop an automated user-friendly device that eliminates the need for third party components.
  - To decrease costs associated with components and permit future high-throughput experiments.

Aim 3
Investigate the mechanical properties of LMS prepared from different layers of the LV wall (Chapter 5).

- To understand whether transmural mechanical heterogeneity exists in the LV wall using a novel intact LMS model.
  - To relate any intrinsic differences in mechanical behaviour observed using LMS to the in vivo operation of the heart.
Chapter Two

General Methods
2 General Methods

This chapter describes the general methodologies used to prepare LMS, perform functional, structural, and molecular experiments, and analyse the acquired data. Methods of specific experiments are described in their respective Chapters. The reagents and equipment used throughout this PhD project are listed here for reference.

2.1 List of reagents

- Isoflurane (CBS, UK)
- Heparin (Fannin, UK)
- 2,3-Butandione Monoxime (2,3-BDM) (Acros Organics, Belgium)
- NaCl (VWR, UK)
- KCl (Sigma-Aldrich, UK)
- Glucose (Sigma-Aldrich, UK)
- HEPES (Sigma-Aldrich, UK)
- MgCl₂ (Sigma-Aldrich, UK)
- CaCl₂ (Sigma-Aldrich, UK)
- Penicillin-Streptomycin (Sigma-Aldrich, UK)
- Insulin-Transferrin-Selenium (ITS) liquid supplement (Sigma-Aldrich, UK)
- L-Ascorbic acid sesquimagnesium salt hydrate (Sigma-Aldrich, UK)
- (-)-Adrenaline (+)-bitartrate salt (Sigma-Aldrich, UK)
- L-(−)-Noradrenaline (+)-bitartrate salt monohydrate (Sigma-Aldrich, UK)
- 3,3′,5′-Triiodo-L-thyronine (Sigma-Aldrich, UK)
- Dexamethasone powder, γ-irradiated (Sigma-Aldrich, UK)
- 1 M BioUltra Ca²⁺ (Sigma-Aldrich, UK)
- Agarose (Sigma-Aldrich, UK)
- Histoacryl surgical glue (Braun, De)
- 100% Ethanol (VWR, UK)
- Polylactic acid (PLA) printing filament (RS Components, UK)
- 95% O₂ 5% CO₂ (BOC, UK)
- 100% medical grade O₂ (BOC, UK)
- Phosphate Buffer Saline (Sigma-Aldrich, UK)
- 16% paraformaldehyde (Alfa aesar, UK)
- Triton X-100 (Sigma-Aldrich, UK)
- SDS-PAGE 4-12% gel (Thermofischer, UK)
- ProQ diamond (Invitrogen, USA)
- SYPRO Ruby ( Invitrogen, USA)
- 100% Methanol (VWR, UK)
- Acetic acid (VWR, UK)
- CellTiter96 (Promega, UK)
- 2 mm stainless steel beads (Qiagen, UK)
- RIPA lysis and extraction buffer (Thermofischer, UK)
- Pierce protease and phosphatase inhibitors (Thermofischer, UK)
- Pierce BCA Kit (Thermofischer, UK)
- 4X Bolt LDS sample buffer (Thermofischer, UK)
- 20X Bolt MES SDS running buffer (Thermofischer, UK)
2.2 List of equipment

- iBlot transfer track polyvinylidene fluoride (PVDF) (Thermofischer, UK)
- Eppendorf 2 ml PCR clean safe-lock tubes (Eppendorf, UK)
- Xylene (VWR, UK)
- 2-mercaptethanol (VWR, UK)
- Bovine serum albumin (BSA) (Sigma-Aldrich, UK)
- Horse serum (Sigma-Aldrich, UK)
- Fetal bovine serum (FBS) (Sigma-Aldrich, UK)
- Pierce 20X TBS Tween buffer (Thermofischer, UK)
- 50 ml Falcon tubes (Corning, UK)
- 2 ml vials (Bertin instruments, FRA)
- Qiazol lysis reagent (Qiagen, DE)
- miRNease mini kit (Qiagen, DE)
- On-Column DNase digestion kit (Qiagen, UK)
- NEBNext® rRNA Depletion Kit (New England Biolabs, USA)
- 203 mm carbon steel scissors (RS Components, UK)
- Forceps (FST, DE)
- Electronic scale
- Spatula
- 60 ml vials (Corning, UK)
- 1 L PYREX bottle (Corning, UK)
- 25 ml Serological Stripette (Corning, UK)
- Stripettor (Corning, UK)
- PIPETMAN Classic P1000, P250, P100, P10, P20 (Gilson, USA)
- 95% CO₂, 5% O₂ (BOC, UK)
- Aluminium foil (Sigma-Aldrich)
- Vibratome (7000 smz2, Campden Instruments Ltd., UK)
- 150 mm x 15 mm Falcon petri dish (Corning, UK)
- Stainless steel blades (VWR, UK)
- Microscissors (FST, DE)
- 3D Printer (Ultimaker, NED)
- 300C dual mode muscle lever (Aurora Scientific, USA)
- LabVIEW (National Instruments, USA)
- PCI I/O. card (National Instruments, USA)
- Peristaltic pump (Masterflex, UK)
- 3/8 in platinum curated peristaltic tubing (Masterflex, UK)
- 3-axis micromanipulator (MCI Ltd, UK)
- Laboratory incubator
- Platinum electrodes (Goodfellow, UK)
- 5 ml sterile Pasteur (VWR, UK)
- Fusion 360 software (Autodesk, USA)
- 18-gauge sterile needles (Sigma-Aldrich)
- Laminar flow hood with UV light
- Pliers (Draper, USA)
- F30 strain gauge (Harvard Apparatus, USA)
- Strain gauge amplifier (Harvard Apparatus, USA)
• MiniDigi digitizer (Molecular devices, USA)
• Axoscope (Molecular devices, USA)
• Video camera (Logitech C920 HD, UK)
• ImageJ (NIH, USA)
• Stainless steel stretchers (custom)
• HeNe Laser (Lasos, DE)
• Rocker
• Confocal microscope (Zeiss and Nikon, UK)
• Typhoon scanner (GE Healthcare, USA)
• 2 mm sterile disposable biopsy puncher (VWR, UK)
• 96-well plates (BIO-RAD, UK)
• TissueLyser LT (Qiagen, UK)
• Centrifuge (Eppendorf, UK)
• Thermocycler (BIO-RAD, UK)
• Electrophoretic cell (BIO-RAD, UK)
• ChemiDoc touch imaging system (BIO-RAD, UK)
• Sequencer (Illumina, USA)
• Prism 8 (GraphPad, USA)

2.3 Animal tissue

LMS were prepared from the hearts of sacrificed animals in accordance with institutional and national regulations, and approved by Imperial College London, under license by the UK Home Office, United Kingdom Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012, and EU directive 2010/63/EU.

2.3.1 Rat

Adult male Sprague-Dawley (SD) rats (12-15 weeks old) (Charles-River, UK) and 300-450 g in weight were sacrificed and LMS prepared according to (Watson et al., 2017). Rats were weighted, placed in animal boxes, and transferred from the animal facility to a designated room in the wet lab. They were anaesthetised using 4% isoflurane at 4 L/min O₂ (CBS, UK). After confirming adequate level of anaesthesia using pedal reflex, rats were removed from the anaesthetic box, and sacrificed via cervical dislocation. The carotid arteries were dissected using 203 mm carbon steel scissors (RS components, UK), confirming death by interruption of blood circulation.

The animal was placed in supine position, and a small incision was made distally on the sternum exposing the xiphoid process. The costal margins and diaphragm were then cut using scissors while holding the xiphoid process with forceps. This exposed the thoracic cavity and permitted visualisation of the heart. The heart and lungs were removed from the animal by cutting across the vena cava, aorta, and connective tissue using scissors and pulling the heart and lungs away from the posterior thoracic wall using the forceps. The heart was never
directly held with the forceps or cut with the scissors; instead, it was always manipulated by holding the lungs.

The extracted heart was placed in a 60 ml vial containing 37 °C heparinised slicing solution (see 2.3.2) and allowed to beat for 5 s to eject any residual ventricular blood. This was done to minimise blood clotting and facilitate the subsequent dissection. It was then immersed in 60 ml vial containing 4 °C heparinised slicing solution and kept there until further dissection.

### 2.3.2 Slicing Tyrode’s solution

The slicing solution is a modified Tyrode’s solution used under the following circumstances, with or without addition of 1000 IU/ml heparin (Fannin, UK):

- Upon removal of the heart from the animal to wash the residual ventricular blood (37 °C, heparinised)
- During transfer of heart from the animal room to the bench for dissection (4 °C, heparinised)
- Whilst preparing the ventricular block (4 °C, non-heparinised)
- During slicing of the ventricular block by the vibratome (4 °C, non-heparinised)
- To maintain the generated LMS quiescent until further experiments (4 °C, non-heparinised)

<table>
<thead>
<tr>
<th>Table 2.1: Slicing solution composition</th>
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<tbody>
<tr>
<td>Solute</td>
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<tr>
<td>--------------</td>
</tr>
<tr>
<td>2-3 BDM</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>KCl</td>
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<tr>
<td>Glucose</td>
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<tr>
<td>HEPES</td>
</tr>
<tr>
<td>MgCl₂</td>
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<tr>
<td>CaCl₂</td>
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The slicing solution was prepared as shown in Table 2.1 (VWR & Sigma-Aldrich, UK and Acros Organics, BE) and titrated to pH = 7. 40 using NaOH measured with a pH meter (Mettler Toledo, UK).
The high BDM and low Ca\textsuperscript{2+} to K\textsuperscript{+} ratio of the slicing solution was chosen to minimise spontaneous contractions of the explanted heart in accordance with previous reports that were performed on rat trabeculae (De Tombe & Little, 1994). Importantly, this solution is different to the one reported in (Watson \textit{et al.}, 2017), which contains 6 mM KCl and 1.8 mM CaCl\textsubscript{2}. It was observed at the beginning of this PhD that the latter did not sufficiently arrest the heart despite effective temperature cooldown. Therefore, the composition of the solution was modified to 9 mM KCl and 1 mM CaCl\textsubscript{2}. This reduced spontaneous contractions, and thus tissue damage during mechanical digestion of the heart with the vibratome (Pitoulis \textit{et al.}, 2019b).

1 L of slicing solution was prepared on each experiment day. Two previously UV-sterilised 60 ml vials (Corning, UK) were filled with slicing solution and supplemented with heparin (1:1000 IU/ml). These were maintained at 4- and 37 °C and were used sequentially and immediately after harvesting the heart as described in 2.3.1. The remainder slicing solution (880 ml) was kept at 4 °C and used during the preparation of the ventricular block (see 2.4.1), the slicing of the ventricular block by the vibratome, and to maintain the generated LMS mechanically quiescent prior to the beginning of experiments.

2.3.3 Normal Tyrode’s solution

For acute functional experiments on freshly prepared slices normal Tyrode’s solution was prepared for each experiment as shown in Table 2.2. This was different to the slicing Tyrode’s solution in its KCl and CaCl\textsubscript{2} concentrations as well as its lack of BDM. The solution was kept at room temperature until the experiments were started.

<table>
<thead>
<tr>
<th>Solute</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>140</td>
</tr>
<tr>
<td>KCl</td>
<td>4.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>10.0</td>
</tr>
<tr>
<td>HEPES</td>
<td>10.0</td>
</tr>
<tr>
<td>MgCl\textsubscript{2}</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCl\textsubscript{2}</td>
<td>1.8</td>
</tr>
</tbody>
</table>
2.3.4 Culture media

For experiments involving chronic culture of LMS (Chapters 3 and 4), Medium-199 with Earle’s Salts (Sigma-Aldrich, UK) was used as culture media. This was supplemented with 3% Penicillin-Streptomycin, 1:1000 Insulin-Transferrin-Selenium, and 20 μg/ml L-Ascorbic Acid (all from Sigma-Aldrich, UK), and a cocktail of four hormones (see 2.3.4.1). During culture, 450 ml of media were exchanged every 24 h, by using a three-way valve system and an autoclaved PYREX 1 L bottle (Corning, UK). Media was not exchanged on the final culture day to avoid disruption of the LMS steady state prior to conduction of experiment. The media was bubbled with 95 % O₂, 5% CO₂ (BOC, UK) to ensure oxygen supplementation of the tissue and maintenance of the pH.

2.3.4.1 Hormonal supplementation of culture media

To maintain tonic neurohormonal drive and mimic the humoral in vivo environment, adrenaline, noradrenaline, triiodothyronine, and dexamethasone were added to the media (All from Sigma-Aldrich, UK). The concentrations of these hormones were chosen to match rat basal plasma levels as determined from the literature (see Table 3.2). For noradrenaline and triiodothyronine in vitro dose-response experiments were also conducted on freshly prepared LMS and the culture concentrations subsequently adjusted (see 3.3.2.1). All hormones were added on the first day, and once every day thereafter, when the culture media was exchanged.

2.4 Preparation of LMS

After harvesting the animal heart, a LV block was prepared, placed in the organ bath of a vibratome stage, and sliced. This yielded the 300μm-thick LMS, which were subsequently trimmed to the required shape and size and had 3D printed rings attached for physical manipulation.

2.4.1 Ventricular block

The term “ventricular block” refers to the isolated, flattened LV that is placed on the vibratome stage for the generation of LMS. The protocol for preparation of the ventricular block has been detailed extensively in (Watson et al., 2017). It is briefly described here. The harvested heart was transferred inside the 60-ml vial containing 4 °C slicing Tyrode’s solution to a dissecting bench and emptied into a 150 mm x 15 mm Falcon petri dish (Corning, UK) pre-filled with 4 °C slicing Tyrode’s solution. A stainless-steel blade (VWR, UK) was used to remove the lungs and any remaining extra-cardiac tissue. A first cut was made superior to the base of the heart using the blade removing the vessel outflow tracts.
This exposed the right ventricular (RV) and LV free wall. The RV free wall was suspended by holding it with forceps against the petri dish wall and removed using microscissors (FST, DE) exposing the interventricular septum. The interventricular septum was then cut across using the microscissors, and the LV propped open. Flattening of the LV is necessary for generation of highly viable LMS as this minimises transection of fibres from different levels of the heart when the vibratome cuts across it (Pitoulis et al., 2019b). To do that, the chordae tendineae, fibrous tissue, as well as any trabeculae that placed undue tension and increased the curvature of the heart were removed using the microscissors. A final cut was made to remove the lateral edges of the interventricular septum using the stainless-steel blade. The flattened ventricular block was then patted dry by gently touching it on tissue roll, and glued using surgical glue (Histoacryl®, Braun, Germany) epicardial side face-down onto a 20 × 20 × 3 mm block of agarose (4% w/v) (Sigma-Aldrich, UK). The agarose was previously glued to the specimen holder using surgical glue. The specimen holder with the attached ventricular block was placed inside the organ bath of the vibratome, filled with 4 ºC slicing Tyrode’s solution, and oxygenated using 100% medical grade O2 (BOC, UK). Slicing of the ventricular block was then initiated. The generated LMS were transferred using a sterile 5 ml Pasteur into a glass container filled with 4 ºC slicing solution and oxygenated with 100% medical grade O2. They were kept there until being trimmed to the required length for future experiments (see 2.4.3).

All dissecting equipment, petri dish, specimen holder, and vibratome equipment were sterilised prior to experiments with 70% v/v ethanol and 30 min under UV light.

2.4.2 Calibration and set-up of vibratome

A high-precision vibrating microtome (7000 smz2, Campden Instruments Ltd., UK) was used to produce the LMS. The vibratome was calibrated for every experiment prior to the ventricular block preparation to a Z-axis error <1.0 μm. To do that, a calibrating device was fitted to its stage, and its ceramic blade cleaned with 70% ethanol. The Z-axis error was then quantified using the vibratome’s user-interface. If the error was > 1.0 μm the blade was cleaned again for a maximum of 3 times with 70% ethanol to remove any dust and/or debris; if the error persisted the blade was changed. This calibration ensured that there was minimal Z-axis movement during slicing of the ventricular block, and by extension minimal damage to the generated LMS.

The vibration settings were:

- 2 mm lateral amplitude
- 80 Hz vibration frequency
- 0.03 mm/s advance speed

These are the optimal setting for production of highly viable LMS (Watson et al., 2017).

2.4.3 Trimming of the LMS

The LMS generated by the vibratome were visualised under a light macroscope. A section of the LMS with most myocardial fibres in a dominant direction was chosen (typically the central part of the LMS) and a first cut was made parallel to the fibres using a stainless-steel blade. Three more cuts were then made each one perpendicular to the previous one, using a millimetre graph paper as guide. The shape of the LMS was thus always rectangular. Its size was measured using digital callipers (RS Components, UK) and used later during analysis to normalise force production to LMS cross-sectional area. The typical LMS size was 10 mm × 8 mm.

2.4.4 Ring attachment

Custom three dimensional (3D) printed rings made from biocompatible polylactic acid (PLA) (RS Components, UK) (Figure 2.1) were attached to the LMS perpendicular to the main fibre axis using surgical glue. The rings permitted physical handling of the LMS and were also used to suspend them between posts, in the bioreactors or in stretchers for fixation, during or after experiments respectively.

2.5 3D printed rings

The rings were designed in Fusion360 (Autodesk, USA) and printed with an Ultimaker Cura 3 printer (Ultimaker, NED) with the main settings as shown in Table 2.3. Several ring designs were used in this project following a series of optimisations for each experimental protocol. The designs and rationale for each are described in Chapter 3.
Table 2.3: Ultimaker 3 required printing parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Print core</td>
<td>AA 0.4</td>
</tr>
<tr>
<td>Layer height</td>
<td>0.05 mm</td>
</tr>
<tr>
<td>Initial layer height</td>
<td>0.1 mm</td>
</tr>
<tr>
<td>Printing temperature</td>
<td>220 °C</td>
</tr>
<tr>
<td>Initial layer temp</td>
<td>215 °C</td>
</tr>
<tr>
<td>Build plate temperature</td>
<td>50 °C</td>
</tr>
<tr>
<td>Print speed</td>
<td>70 mm/s</td>
</tr>
<tr>
<td>Plate adhesion</td>
<td>No</td>
</tr>
<tr>
<td>Cooling fan power</td>
<td>100 %</td>
</tr>
</tbody>
</table>

2.6 *In vitro* culture of LMS

Two culture set-ups were used for chronic *in vitro* experiments. The first was developed in collaboration with Prof Pieter de Tombe and utilised as its main component the 300C dual-mode muscle lever from Aurora Scientific, USA. Henceforth, this will be referred to as the Aurora. Its operation is described in detail in Chapter 3. The other, MyoLoop, was developed during this PhD. MyoLoop introduced many novelties and was designed, prototyped, and created by us from scratch. The motivation, R&D, and its technical aspects are therefore described extensively in Chapter 4. Although the protocols for mounting LMS on either set-up were very similar in both, each is described in the respective chapter – that is, Chapter 3 for Aurora, and Chapter 4 for MyoLoop.

2.7 Functional experiments

Functional characterisation of LMS was investigated by performing force-stretch experiments and analysing the active and passive force curves, by characterisation of the LMS contractile state using a modified time-varying elastance model, and by force-Ca\(^{2+}\), force-noradrenaline, and force-triiodothyronine dose-response experiments. The methodology for the force-stretch responses of the chronic *in vitro* experiments of Chapter 3 and Chapter 4, as well as the acute experiments on fresh LMS of Chapter 5 was common and thus described here. Force-noradrenaline and force-triiodothyronine experiments are described in Chapter 3 (3.2.3) whereas force-Ca\(^{2+}\) experiments are discussed in Chapter 5 (5.2.4).
2.7.1 Measuring force production of fresh and cultured LMS

Isometric Frank-Starling, and force-dose response experiments were conducted in fresh and cultured LMS during this PhD. These experiments were performed with the LMS immersed in an organ bath or in the Aurora. The general methodology is described here and the exact protocols in Chapters 3 (3.2.3 & 3.2.10) and Chapters 5 (5.2.4 & 5.2.5).

On fresh LMS, the experiments were conducted in an organ bath set-up designed for acute experiments (Figure 2.1). This consisted of a 15 mm petri dish bath, an isometric F30 strain-gauge (Harvard Apparatus, USA) mounted on a micromanipulator (Harvard Apparatus, USA), temperature-controlled perfusion, and platinum electrodes connected to a voltage-controlled biphasic field stimulator (IonOptix, IE) (Figure 2.1). Force data were acquired continuously (free scope mode) using an amplifier (Harvard Apparatus, USA), a MiniDigi digitizer (Molecular Devices, USA) and the Axoscope software (Molecular Devices, USA) at 1 kHz sampling frequency. For these experiments, the SL of LMS was used to control the preload. To do that, a video camera (Logitech C920 HD, UK) was positioned vertically superior to the organ bath, connected to a portable computer, and calibrated with the ImageJ software webcam plugin (NIH, USA) to a known distance on the petri dish. This was then used to measured muscle length in real-time with ± 0.1 mm accuracy. The required preload was then set by stretching the LMS to the desired muscle length, given a previously calculated SL-% stretch relationship (2.8.1). Acquired force data were subsequently analysed manually using the pClamp software package (Molecular Devices, USA) as described in 2.10.1.1.

For cultured LMS, the experiments were conducted in situ, in the Aurora or MyoLoop and not in the organ bath shown in Figure 2.1. This followed early observations that transfer of cultured LMS to the organ bath, as previously done in our lab (Watson et al., 2019), caused rapid contracture and/or tachyarrhythmias coupled with quick run-down in force. It was hypothesised that this was most likely due to differences in osmolarity and solute composition between the Tyrode’s solution, used in the organ bath, and the culture media, used to culture the LMS in the Aurora. Thus, to avoid disruptions to the LMS steady state and experimental artefacts all functional experiments after culture were performed in the Aurora.
Figure 2.1: Organ bath used for acute experiments and isometric Frank-Starling trace

A) Zoomed in image of organ bath. The LMS are mounted between isometric strain gauge-micromanipulator (right) module and fixed post (left). This set-up was used for acute Frank-Starling, force-Ca2+, force-noradrenaline, and force-triiodothyronine experiments. Electrodes and perfusion inflow/outflow lie laterally to the LMS and are not shown here. Temperature is controlled by recirculation bath B) Representative Frank-Starling trace. Panel A obtained from (Pitoulis et al., 2020) under CCL.
2.8 Structural experiments

2.8.1 Quantification of sarcomere length with laser diffraction

Laser diffraction was used on freshly prepared LMS to correlate SL with muscle length. These experiments yielded a SL-% stretch relationship, which was then used to stretch LMS to the desired SL using muscle length as a surrogate marker. This was a) less technically challenging than performing laser diffraction on every LMS, and b) more physiologically relevant than using a % amount of stretch without an understanding of how this correlated to the preparation’s SL. Ultimately, the data obtained from these studies were used to stretch LMS for both acute and chronic experiments.

LMS were mounted on custom-made stainless steel stretchers (Watson et al., 2019) by manipulating them from their rings. The stretchers which are described in 1.2.4 and shown in Figure 1.9 allow manipulation of the LMS SL via the use of a hex-screw. After mounting the LMS, the stretcher was placed into a glass petri dish previously filled with slicing Tyrode’s solution at room temperature. A high powered HeNe laser (Lasos, Germany) was positioned 2 cm vertically above the petri dish, using a clamping rod as described in (Watson et al., 2019) and (Pitoulis et al., 2020). The laser was then turned on passing through the LMS and diffracting into a series of bands. The distance between the bands depends on the SL of the preparation (Cleworth & Edman, 1969). The diffraction profiles were captured with a calibrated camera (Logitech C920 HD, UK) situated superiorly to the bands and analysed in real-time using the intensity profile functionality of ImageJ as detailed in (Watson et al., 2019). The LMS were then progressively stretched to three different muscle lengths, and data acquired at each length. As SL can exhibit heterogeneity within the same preparation (Pitoulis et al., 2020), for each % stretch, the laser was passed at three different LMS regions and diffraction data obtained for each. These were then averaged, and a linear relationship was fit to the SL-% stretch data as described in 2.10.5.

The calculated SL-% stretch relationship was used to set the preload in the acute isometric Frank-Starling (5.2.5), dose-response experiments (constant preload – see 3.2.3 and 5.2.4) and setting the preload at the beginning of culture in the Aurora (3.2.8) and MyoLoop (4.9.1.1).

2.8.2 Immunohistochemistry

LMS were mounted on stretchers, stretched to 110 % stretch, and fixed in 4 % formaldehyde diluted in phosphate buffered solution (PBS) (Sigma-Aldrich, UK), for 15 min at room temperature. They were then washed in PBS and permeabilised and blocked with
1.5% Triton X-100 (Sigma, UK) diluted in 10% FBS, 5% BSA and 10% horse serum (All from Sigma, UK) and placed on a rocker at room temperature. After three hours, the LMS were washed in PBS three times, incubated with primary antibodies, and left overnight at room temperature on a rocker. They were washed again three times in PBS, incubated with the secondary antibodies for 2 hours at room temperature on a rocker and covered with aluminium foil to prevent photobleaching. The secondary antibodies were washed off with PBS three times, and the samples stored at 4 ºC in PBS until visualisation under confocal microscopy. For visualisation of cellular membranes with wheat germin agglutin (WGA), and DNA with Hoechst-33347, LMS were stained by placing them in the respective dye for 15 min at 3 μg/ml and 1:1000 dilutions respectively. As the latter were conjugates, secondary antibodies were not required. All LMS were visualised within 1 day of being stained.

Table 2.4: Primary and secondary antibodies or dyes and respective experiments for which they were used

<table>
<thead>
<tr>
<th>1º Antibodies or Dye</th>
<th>Dilution</th>
<th>2º Antibodies</th>
<th>Dilution</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse caveolin-3</td>
<td>1:500</td>
<td>Donkey anti-mouse Alexa Fluor 546</td>
<td>1:2000</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>Rabbit cardiac troponin T</td>
<td>1:800</td>
<td>Donkey, anti-rabbit Alexa Fluor 488</td>
<td>1:2000</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>Chicken vimentin</td>
<td>1:3000</td>
<td>Goat, anti-chicken Alexa Fluor 647</td>
<td>1:2000</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>WGA</td>
<td>3 μg/ml</td>
<td>-</td>
<td>-</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Hoechst-33347</td>
<td>1:1000</td>
<td>-</td>
<td>-</td>
<td>Chapter 5</td>
</tr>
</tbody>
</table>

2.8.3 Confocal microscopy

A confocal microscope (Zeiss LSM-780, UK) was used for visualisation of stained LMS. For assessment of cardiomyocyte dimensions (area, width, length), Z-stack images were obtained at 20x magnification using caveolin-3 for fresh LMS (Chapter 5), and WGA for cultured LMS (Chapter 3). WGA was used in cultured LMS as caveolin-3 staining did not stain the membranes sufficiently making quantification of cardiomyocyte dimensions difficult. 3 and 5 Z-stack images were obtained per LMS for each experiment respectively. Z-stacks were obtained until the thickness of the tissue made further visualisation impossible.
2.9 Molecular characterisation of LMS

The molecular phenotype of LMS was probed in different modalities based on experimental hypothesis. For experiments in Chapter 5, ProQ Diamond and Sypro Ruby stain was done to compare differences sarcomeric protein content and phosphorylation of LMS from different layers of the ventricular wall. For experiments in Chapter 3, we performed measurements of LMS bath pH, LMS viability, western blots, and RNA sequencing. These methodologies are described in their respective chapters.

2.10 Data Analysis

This section discusses general techniques used for analysis of functional, structural, and molecular data acquired during this project. Chapter-specific techniques (e.g. time-varying elastance in Chapter 3) are discussed in their respective chapters.

2.10.1 Analysis of LMS functional data

The function of LMS was characterised by analysing force transients of LMS under different experimental conditions (Frank-Starling, force-Ca\(^{2+}\), force-noradrenaline, and force-triiodothyronine dose-response experiments). Two different approaches were used for these. At the beginning of this PhD, the standard method of the lab, involving manual analysis (2.10.1.1) was used. During this PhD, custom LabVIEW (National Instruments, USA) programs were written to analyse force-traces and subsequently to a) minimise time spent on analysis, and b) remove potential bias. The reason behind using different methods of analysis was the different timeframe of the projects – that is, experiments of Chapter 5 preceded experiments of Chapter 3 and hence the development of the LabVIEW programs.

2.10.1.1 Manual Analysis

Manual analysis was performed with Clampfit (Molecular Devices, USA). The method described here was used for the Frank-Starling and force-Ca\(^{2+}\) experiments reported in Chapter 5 and force-noradrenaline, and force-triiodothyronine experiments reported in Chapter 3.

For each dose or SL three consecutive force transients at steady state were analysed as follows. Steady state was defined as non-fluctuating twitch force baseline and amplitude for at least 30 s. A transient was selected using the Clampfit software’s cursors. The ‘Statistics’ command was then used with ‘Peak amplitude’, and ‘Baseline’ checkbox measurements selected. This yielded the peak force and baseline of the selected transient. The amplitude corresponding to active tension was calculated by subtracting the baseline from peak force.
This process was repeated for all three transients. Values were normalised to the LMS cross-sectional area (LMS thickness (0.3 mm) × slice width (mm)).

**2.10.1.2 Automatic analysis**

For the Frank-Starling data described in Chapter 3, custom LabVIEW programs were developed. These automatically determined the active tension and baseline force of any number of consecutive transients (Figure 2.2). All values were normalised to the LMS cross-sectional area automatically by inputting the LMS width in the program. The program accepted a .txt or .csv file of a force transient and outputted the analysed data in .txt column format.

**2.10.2 Linear regressions of active force-SL and active force-% stretch**

A linear regression was fit to the active force-SL of the experiments in Chapter 5 and active force-% stretch of the experiments in Chapter 3. This allowed us to determine mechanical output per unit stretch, with the slope of each line equal to $dF/d\mu m$ or $dF/d\%$ respectively and providing a numerical value of the sensitivity of LMS to stretch. Linear regression models were fit using Prism 8 (GraphPad, USA).

**2.10.3 Force-Ca$^{2+}$, force-noradrenaline, and force-triiodothyronine**

The force generated by LMS at different Ca$^{2+}$ and noradrenaline or triiodothyronine concentrations was quantified manually as described before. The active-force was then plotted against a log scale of [Ca$^{2+}$] or [noradrenaline/triiodothyronine] and a sigmoidal log(agonist) vs. response (variable slope with four parameters) model was fitted according to the equation:

$$A = B + C \frac{1}{1 + 10^{((\log EC_{50} - \log dose)) + H}}$$

where $A$ is the active-force, $B$ is the passive force, $C$ is the max developed force, $H$ is the hill coefficient, and $EC_{50}$ is the [Ca$^{2+}$], [noradrenaline], or [triiodothyronine] required to reach half maximum active-force. The model was fit to the data in Prism 8.
Figure 2.2: LabVIEW program developed during this thesis for automatic analysis of force transients
A) Front panel (user-interface) of program. Data files are loaded as .csv or .txt file and the program is then run. It can analyse any number of transients by enclosing them between the two cursors (orange and purple). Pressing the ‘Log Data’ button will automatically calculate the amplitude and baseline of the selected transients. Kinetics can also be determined using an additional Boolean module but are not shown here. B) [QR code](#) of the program in action (password: phd?)
2.10.4 Immunohistochemistry

Images acquired from confocal microscopy were used to evaluate the structural characteristics of LMS. All reviewers were blinded, and analysis performed in Image J. One reviewer (Waseem Hasan) analysed the images for the experiments in Chapter 5, and two reviewers (Dr Worrapong, Kit-Anan, and Prof Cesare Terracciano) analysed data for experiments in Chapter 3.

2.10.4.1 Cardiomyocyte area

For Chapter 3, cardiomyocyte area was calculated by circumscribing along the membrane defined by the caveolin-3. The length of the cell was defined as the longest axis across a cardiomyocyte and width as the longest line perpendicular to the length axis. For chronic experiments (Chapter 3) cardiomyocyte area was quantified by circumscribing a rectangle along the cardiomyocyte borders demarcated by the fluorescence WGA. Both were done on confocal images obtained at x20 magnification. 12 and 8 cells were analysed per image for each experiment respectively.

2.10.4.2 Cardiomyocyte length, width, and aspect ratio

The length, and width of each perimetrical or rectangular circumscription corresponded to the cardiomyocyte’s length, and width and the aspect ratio was calculated as:

\[
\text{Cardiomyocyte aspect ratio} = \frac{\text{length}}{\text{width}}
\]  

(10)

2.10.4.3 Cardiomyocyte density

The number of fully visible cardiomyocytes in an image, at x20 magnification, was used to determine cardiomyocyte density. This was done only for the transmural experiments described in Chapter 5.

2.10.4.4 Total cardiomyocyte area

Total myocyte area was defined as the total area of an LMS covered by myocytes. It was calculated for the experiments of Chapter 5 as:

\[
\text{Total cardiomyocyte area} = \text{cell density} \times \text{cardiomyocyte area}
\]  

(11)

2.10.5 Laser diffraction

The SL-% stretch data acquired from the laser diffraction experiments was fit with a linear regression. For the experiments in Chapter 5, a linear regression was fit to the data of each cardiac layer (see Figure 5.2). This was then used to stretch LMS from different layers.
of the ventricle to the required SL. Subsequently, the data from all layers were pooled and a single SL-% stretch relationship for LMS independent of position in ventricular wall region was obtained. This was used to stretch LMS to the desired preload during the chronic experiments as described in (3.2.8). Linear regressions were fit using Prism 8.

2.11 Programming in Python and LabVIEW

Multiple computer programs and algorithms were developed throughout this PhD to facilitate with data analysis, data visualisation, as well as for control, and operation of MyoLoop (Chapter 4). The source code of a number of these is available in the Appendix and is protected by a patent by Imperial College London – Patent ID 10482. All first versions of LabVIEW programs were written in LabVIEW 8 in a 2013 MacBook Pro that was running Windows 10 in bootcamp mode, before being packaged into standalone LabVIEW executable application for device dissemination. All Python codes were written in Atom (MIT, USA) text editor in a 2017 MacBook pro running macOS Catalina. The main programs are summarised here.

- **LabVIEW transient analyser**: Automatically analyses any number of transients for amplitude, baseline, and kinetics. Output data are saved as .txt file in user specified path. Described in 2.10.1.2.

- **LabVIEW elastance calculator**: Runs a modified time-varying elastance theory on LMS force-length data. Accepts any number of work loops (≥ 2) and outputs a sequence of isochrones and the maximum time-varying elastance, \( E_{\text{max}} \). Output data are saved as .txt file in user specified path. Described in detail in 3.2.9.

- **LabVIEW MyoLoop front & backend**: User interface and algorithms for MyoLoop operation including control of data acquisition, actuator position, real-time analysis of acquired data, all mathematical models, and general I/O of the MyoLoop device. Described in detail in Chapter 4.

- **Python MyoLoop control algorithms**: Python programs that connect to and control the custom peripheral hardware of MyoLoop were developed. These included modules for the MyoLoop stimulator, amplifier, temperature control and simple user interface for parameter adjustment of these. Sample codes of these are provided in the Appendix and described in detail in Chapter 4.
2.12 Circuit design and soldering

All hardware circuits for MyoLoop (Chapter 4) were designed in the web-based circuit designer EasyEDA. Components for rapid electrical prototyping (on breadboards) were ordered from RS components, DigiKey, and RaspberryPI Hut, all from UK. The final MyoLoop PCB was ordered from JLCPCB, CHN. All soldering was done using a Holife iron station with 0.3 mm lead-free 99.3 % Sn 0.7% Cu 2.2% rosin core flux solder.

2.13 Statistics

Statistical analysis was performed in Prism 8. First, D’Agostino Pearson and Shapiro-Wilk test were used for assessment of normality. If data sets passed the normality test, then one-way and two-way analysis of variance (ANOVA) with Tukey’s post-hoc was used for determination of statistical differences between group means. For non-normally distributed data sets, Kruskal-Wallis was used. For statistical comparison of linear regressions, the slopes were compared using analysis of covariance (ANCOVA) as detailed in (GraphPad Prism, 2017) and described before in (Pitoulis et al., 2020). For data sets with multiple technical replicates per biological sample and unequal sample distributions hierarchical (nested) ANOVA was used (e.g. in confocal analysis of cardiomyocyte morphology). P<0.05 was considered statistically significant. The statistics are described in the methods section of each chapter respectively.

From previous experiments we have calculated that for a 35% change in LMS force of contraction, a sample of n = 5 LMS biological replicates yields a power of 0.81. As such, 5-6 LMS from different rats were used per group per experiment.
Chapter Three

Advanced electromechanical cardiac tissue culture to recreate load-induced remodelling \textit{in vitro}
3 Advanced electromechanical cardiac tissue culture to recreate load-induced remodelling in vitro

3.1 Introduction

In vitro cardiac models are an indispensable tool for basic cardiovascular research. They are more reductionist than their in vivo counterparts as the effects of interacting systems present in vivo (e.g. neurohormonal axis, inflammatory pathways) are minimised, allowing for greater degrees of causality between dependent and independent variables (Pitoulis et al., 2019b). However, because of their frequently oversimplified nature and distance to physiology, the results obtained in vitro often fail to translate.

Physiological relevance is particularly important for chronic in vitro studies such as culture of cardiac tissue. The heart is extremely sophisticated and incessantly exposed to electrical and mechanical stimulation as well as complex extrinsic and intrinsic feedback loops. Thus, when adult cardiac tissue is cultured in absence of these elements in vitro it rapidly remodels, losing its archetypical structure and function (Fischer et al., 2019)(Watson et al., 2019)(Louch, Sheehan & Wolska, 2011)(Zhang et al., 2010).

Consequently, biomimetic techniques (see 1.2.4) that recreate the in vivo environment in vitro have proliferated in the field of cardiac tissue engineering in the last decades. Such approaches range from mimicking the cardiac hormonal environment (Yang et al., 2014b), to intricate pacing protocols (Ronaldson-Bouchard et al., 2018), to mechanically loading tissues (Watson et al., 2019) (Fischer et al., 2019). The latter has received special attention, as mechanical load is a determinant of both acute (Pitoulis & de Tombe, 2019) and long term (Pitoulis & Terracciano, 2020) cardiac performance. The heart is always exposed to haemodynamic load and can develop radically different phenotypes depending on the nature and time-course of exposure (Mihl, Dassen & Kuipers, 2008)(Ibrahim et al., 2010)(Zimmermann, 2013). For example, pressure-overload, as is seen in aortic stenosis and hypertension, induces characteristic concentric remodelling with initially compensated but progressively deteriorating function (Mihl, Dassen & Kuipers, 2008). In contrast, volume-overload, seen with valvular insufficiency, is associated with eccentric remodelling (Pitoulis & Terracciano, 2020) (see also 1.1.3)

All protocols that attempt to simulate load in vitro involve stretching and or relaxing cardiac tissue during culture; the difference between protocols lies in the stretch/relaxation pattern, and the extent to which this accurately recapitulates the mechanical load experienced by the in vivo beating heart. Currently, the gold-standard method to culture cardiac muscle in
**vitro** with mechanical load is to mount it on flexible posts that bend upon tissue force development, as described in (Fischer et al., 2019), (Ronaldson-Bouchard et al., 2018), and (Mannhardt et al., 2019). Under these conditions the tissue is beating in an auxotonic mode of contraction, shortening against the flexible posts upon electrical stimulation. The stiffness of the post can be adjusted to increase or decrease afterload, while preload is set by stretching the tissue to the desired diastolic length (Fischer et al., 2019). The approach used extensively by our lab in the past (Perbellini et al., 2018b)(Watson et al., 2019) and more recently others (Ou et al., 2019), involves culturing cardiac tissue on fixed inflexible posts, which do not bend upon tissue contraction – that is, the tissue contracts isometrically (see Figure 1.8 and Figure 3.1 for comparison of auxotonic and isometric).

These approaches fail to recreate the dynamic sequence of mechanical actions of the *in vivo* cardiac cycle, which consists of discrete phases of isometric contraction, ejection, isometric relaxation, and diastolic refilling (Figure 1.8 and 1.2.4.1). Under auxotonic load, the length and force have a linear relationship like an elastic band. Likewise, although technically straightforward, isometric contractions are *de facto* against infinite and thus highly unphysiological afterload. Thus, regardless of whether the *in vitro* cardiac model is an LMS or an EHT, these approaches limit physiological relevance and translational capacity.

In this chapter, we aimed to develop a novel methodology to simulate the *in vivo* cardiac cycle on LMS during *in vitro* culture. The objectives were:

a. Develop an advanced electromechanical *in vitro* culture model that approximates the physiological cardiac cycle

b. Use the method developed in (a) to chronically simulate physiological and pathological load profiles on LMS for the study of load-induced remodelling *in vitro*

To address objective (a) we developed a platform to culture LMS under continuous mechanical load that recreated all phases of the cardiac cycle. To do that, we used a 3EWK model (1.2.4.2) that controlled the length of the LMS in sync with electrical stimulation via custom analog-digital LabVIEW algorithms. With this approach, LMS afterload was controlled by the 3EWK variables, namely: arterial impedance (Ra), peripheral resistance (Rc), and arterial compliance (Ca); all of which have circulatory physiological relevance (Westerhof, Lankhaar & Westerhof, 2009), while the preload was controlled by stretching the LMS to the desired diastolic SL, using the LMS muscle length as a surrogate marker, at the beginning of culture.
To address objective (b) we used the platform and methodology developed in (a) to chronically expose LMS to mechanical load simulating physiological load, pressure-, or volume-overload for 3 days. We then performed temporal and terminal functional, structural, and molecular phenotyping to determine the effects of load on myocardial remodelling *in vitro*.
Figure 3.1: Approaches to mechanically load cardiac tissue in vitro
A) Cartoon schematic cardiac mechanical behaviour during systole and diastole under isometric, auxotonic, or physiological load. B) Phase plane force-length work loops based on previously simulated data of the different loads. Figure adapted from (Pitoulis et al., 2021) under CCL.
3.2 Methods

3.2.1 LMS preparation

LMS were prepared from adult SD rats, trimmed, and had rings attached perpendicular to the main myocardial fibre direction as described in 2.4.

3.2.2 Laser diffraction experiments

The SL-% stretch relationship of fresh LMS was quantified and analysed as described in 2.8.1 and 2.10.5 respectively. This relationship was used together with the LMS resting length to stretch the LMS to desired SL, and thus preload, during the beginning of the experiments by using the muscle length as a surrogate of SL.

3.2.3 Noradrenaline-triiodothyronine dose-response curves

The sensitivity of LMS to noradrenaline was determined by performing a 6-point half-log dose-response (force) curve. The noradrenaline concentrations (control, 0.25-, 2.5-, 25-, 250-, and 2500 nM) were prepared by diluting L-(-)-Noradrenaline (+)-bitartrate salt monohydrate in normal Tyrode’s solutions with 0.1 mg/ml L-ascorbic acid. The solutions were kept in 500 ml glass bottles at room temperature and covered with aluminium foil prior, and during the experiments. Triiodothyronine dose-response were performed with the same concentrations as noradrenaline by Dr Raquel Nunez-Toldra.

The solutions were transferred to the organ bath strain gauge set-up described in 2.7.1 and shown in Figure 2.1, connected to the perfusion system, oxygenated, and kept at 25 ºC. Prepared LMS were then transferred to the organ bath, mounted between the strain gauge and micromanipulator, perfusion with the control solution (no hormone) started, the temperature of the bath slowly raised from 25 ºC to 36 ± 0.5 ºC, and the electrical stimulation set to 1 Hz, 20-30 V, 10-20 ms pulse width. The mounted LMS was then progressively stretched to 2.2 μm SL over ~ 7 min using a video camera measuring muscle length in real-time as described in 2.7.1. LMS were kept at this preload for the entire dose-response experiment. After 10 min of perfusion with the control solution an unused concentration of noradrenaline or triiodothyronine was chosen at random and allowed to perfuse the LMS for 10 min or until steady state. This process was repeated for all hormone concentrations. Data were analysed as described in 2.10.1.1 and the force-dose curve was fitted as described in 2.10.3 and equation (9).
3.2.4 Culture media

Culture media was prepared fresh at the start of each experiment; media was replaced on each culture day as described in 2.3.4.

3.2.5 Rings

3D printed rings were printed as described in 2.5. Different ring designs were developed for culture of LMS. The rationale behind each is described in 3.3.2.2.

3.2.6 Development of the Aurora set-up for culture of LMS in vitro

The Aurora was developed as a proof-of-concept platform to recreate the mechanical events of the cardiac cycle in vitro. It consisted of the 300C dual mode muscle lever (Aurora Scientific, USA), which can simultaneously measure force and lengthen/shorten tissue, a custom culture chamber, a MasterFlex peristaltic pump and 3/8 in platinum-curated peristaltic tubing (Cole-Parmer, UK), an incubator, a 500 ml autoclavable bottle which stored the culture media, and a C-Pace stimulator (IonOptix, IE). The 300C muscle lever was controlled by custom written LabVIEW (National Instruments, USA) algorithms deployed on a portable computer (Dell, USA) with a multifunctional (analog-to-digital and digital-to-analog) I/O PCI card installed (National Instruments, USA). The Aurora set-up is shown in Figure 3.2.

The culture chamber was designed in Fusion 360 (Autodesk, USA) and made from Ertacetal-C using a CNC-drill in the Imperial College Advanced Hackspace facilities. 0.3 mm clearance holes were drilled at opposite ends to allow for electrical field pacing via 0.2 mm platinum electrodes (Goodfellow, UK), which were connected to the stimulator. The 300C was suspended superior to the culture chamber by fitting it into a custom 3D-printed holding device (Figure 3.2). The steel part of an 18-gauge needle (Sigma-Aldrich, UK) was manually bent using pliers (Draper, USA) and glued on the lower side of the 300C lever using surgical glue. On the opposite side another L-shaped needle was fixed on a rod attached to a 3-axis micromanipulator (MCI Ltd, UK). The two needles projected inferiorly into the culture chamber and were used to suspend the LMS from its rings during culture and can be seen in the red rectangular showing where the LMS is suspended on Figure 3.2. The micromanipulator allowed adjustments in the diastolic length of the LMS. Due to the nature of the setup, there was no chamber lid during culture. Culture media was recirculated using a double perfusion system (bottle to chamber to bottle). It consisted of the peristaltic pump, reservoir media in the bottle, and inflow & outflow tubing, and it was used as it allowed us to have 500 ml of reservoir media in the bottle during culture. Pump speed was set to 80 ml/min. Media was supplied with O₂ and its pH kept constant at ~ 7.40 by perfusing the
bottle with 95% O₂, 5% CO₂. The whole set-up was placed in an incubator and kept at 37 ºC (Figure 3.2).

Prior to any culture experiments in the Aurora, the media bottle, culture chamber, and peristaltic pump tubing were autoclaved and then handled in sterile manner under laminar flow hood. The incubator, as well as the needles for LMS suspension, which were permanently attached to the 300C, and micromanipulator rod were disinfected with 70% ethanol before and after every experiment. The bottle was filled with media ~ 45 min before mounting the first LMS to the Aurora to ensure that the desired temperature had been reached. The steps describing the mounting of LMS in the Aurora are described in 3.2.8. These steps assume that the LMS are trimmed and have rings attached already.
**Figure 3.2: The Aurora set-up**

A) The Aurora set-up used for chronic in vitro culture of LMS under a 3EWK model. The media bottle was kept behind the 3D printed 300C holder during culture (not shown here), while the stimulator and peristaltic pump are positioned outside the incubator (not shown here). B) Cartoon schematic of an LMS suspended in the Aurora. C) **QR code** showing the CNC machining of the culture chamber at Imperial Advanced Hackspace (password: phd?).
3.2.7 Aurora algorithms

Custom LabVIEW programs were developed in collaboration with Prof Pieter de Tombe to control the LMS muscle using the 300C. These permitted mechanical loading of the LMS during culture based on a 3EWK model. The 3EWK controlled the LMS rate and amount of shortening (stroke length) during the systolic phase of a twitch. To do that a length transient was first predicted by the software based on the 3EWK parameters (Ra, Rc, Ca) chosen by the user and the force generated by the LMS and acquired by the 300C. The length-transient was then actuated in sync with the electrical stimulation using a proportional-integral-derivative (PID) algorithm that enabled convergence of the predicted and actuated length-transients over a 30 s period, as has been previously described (De Tombe & Little, 1994).

The Aurora sampled 512 ms of each LMS force transient at 1 kHz sampling frequency. The acquisition was triggered by a digital rising transistor-transistor logic (TTL) from the C-pace stimulator. The acquired force was fed to a module in the LabVIEW software that run a 3EWK model. As the 3EWK was numerically solved in the pressure-volume state space, the force data were first converted to pressure using Laplace’s law of the heart (equation (3)) (Grossman, Jones & McLaurin, 1975). The output of the model was a LV volume transient which was converted to length using a spherical model of the LV and solving for radius (De Tombe & Little, 1994). The predicted length transient was then actuated on the LMS using a PID module as aforementioned. In summary, an input force-transient was run through a 3EWK algorithm the predictions of which were then actuated in real time on the LMS.

The diastolic re-stretching phase was kept the same for all LMS cultured in the Aurora. It was set to a linear ramp from end-systolic to end-diastolic length completed within a 200 ms period at the end of the 512 ms data acquisition (Figure 3.3). Data from the diastolic refilling phase were not acquired and thus force-length loops obtained in the Aurora are shown with an open bottom (Figure 3.3). This was done to minimise computational demand.

Ultimately, the Aurora enabled us to recreate the pressure-volume changes of the in vivo myocardium in vitro at the force-length plane by shortening the LMS during systole and re-stretching during diastole (Figure 3.3). Importantly, the length transients predicted by the 3EWK depended on the force generated by the LMS and the process of force transient acquisition and length-transient prediction was performed for every beat during culture. Therefore, a change in force production was accompanied by a change in length-transient, making our bioreactor adaptive to the remodelling LMS. The advantages of this feature are discussed in 3.3.4.2 below.
Figure 3.3: Aurora mode of operation
A) 512 ms of a force transient of a beating LMS were acquired and fed to a 3EWK model. The 3EWK predicted a volume transient, which was converted to length using a spherical model of the LV. This was then applied to the LMS. B) Representative force-length work loop of a LMS beating in the Aurora. C) QR code of LMS ‘looping’ in the Aurora set-up (password: phd?).
3.2.8 Culture of LMS in the Aurora

Prepared LMS were briefly rinsed (~ 10 s) with culture media using a sterile Pasteur at room temperature; this removed excess slicing Tyrode’s solution. They were then transferred to the Aurora setup and suspended between the 300C and micromanipulator needles using forceps. Prior to mounting, the micromanipulator was positioned such that the two opposite needles were close enough to allow for LMS suspension without undue tissue stretching. Preload was controlled by stretching the LMS using the micromanipulator and digital callipers to the desired SL. The desired SL was reached by stretching based on the LMS resting muscle length (RL), and the SL-% stretch relationships (see Figure 3.7 and equation (13)). Initially, the LMS were stretched to 105% of their RL and kept at this length for 15 min. The peristaltic pump was started and once the LMS was immersed in media the stimulator was set to 1 Hz, 6–15 V pulse amplitude and 5-10 ms pulse width. The LabVIEW program controlling the 300C dual mode muscle lever was run and data acquisition initiated. After 15 min at 105% RL, LMS were mechanically loaded to the desired preload and afterload based on experimental condition – that is, isometric load for objective (a), and physiological load, pressure-, or volume-overload for objective (b). Once set these parameters were not altered for the duration of culture.

For isometric load, physiological load, and pressure-overload this was set to 2.1 μm SL, corresponded to 110% of RL, as determined from laser diffraction experiments. For volume-overload, LMS were stretched to a preload of 2.3 μm, equivalent to 126% stretch from RL.

For physiological load, pressure-overload, and volume-overload, afterload was set using the parameters of the 3EWK model (Ra, Rc, Ca) available in the LabVIEW program user-interface. It was set to reflect physiological (Ra = 7, Ca = 0.5, Rc = 0.5) in physiological and volume-overload, or pathologically high afterload (Ra = 10, Ca = 0.3, Rc = 0.9) in pressure-overload. For isometric load the 3EWK was not used to control afterload, as the 300C was clamped to a fixed position, preventing any LMS shortening.

The rationale behind the chosen parameters of preload and afterload is discussed below and summarised in Table 3.3

3.2.9 Assessment of the load-independent contractile state of cultured LMS

For the LMS cultured under physiological load, pressure-overload, or volume-overload, we developed a protocol and a LabVIEW algorithm to assess the load-independent intrinsic contractile state of LMS throughout culture. This was based on the theory of time-varying elastance ($E_t$) (Suga, Sagawa & Shoukas, 1973), and the rationale behind it is explained in
3.3.4.3. Only the protocols for data acquisition and computer algorithms for analysis are described here.

On each culture day, all LMS, irrespective of their loading condition, were made to do the same 4 work-loops. These had 3EWK parameters that covered a spectrum of systolic loads (1. Ra = 7, Ca = 0.5, Rc = 0.5, 2. Ra = 5, Ca = 0.7, Rc = 0.2, 3. Ra = 10, Ca = 0.3, Rc = 0.9, and 4. Ra = 5, Ca = 0.5, Rc = 0.5) in order to increase the reliability of the time-varying elastance fitting on the data. In principle, only two work loops are needed to assess $E_t$, given that the model uses a sequence of linear regressions to determine contractility, however, four were used here. The force and length-transients of each work loop were acquired and stored for offline analysis. The whole protocol lasted ~5 min.

The developed LabVIEW program accepted any number of work loops as input and outputted a sequence of isochrones, the $E_t$ transient, and the maximum elastance ($E_{max}$) (Figure 3.4). Each isochrone is a linear regression that fits to data from the 4 different work loops that occur at the same time (ms) of the cardiac cycle. Then, the slope of each isochrone corresponds to one data point of the $E_t$ transient, and the amplitude of $E_t$ corresponds to $E_{max}$, the load-independent measure of intrinsic tissue contractility. In the original theory, dead volume ($V_d$) constrains the isochrones on the x-axis and corresponds to the ventricular volume below which no pressure generation occurs (Sagawa, 1981). As force and length data were used here, we modified $V_d$ to $RL$, that is the muscle length below which no force generation occurs. The equation that describes this is the following:

$$E_t = \frac{F(t)}{L(t) - RL}$$  \hspace{1cm} (12)  

Where $F(t)$, and $L(t)$ are the force and length of an LMS at any given time during the cardiac cycle.
Figure 3.4: Modified time-varying elastance ($E_t$) for assessment of intrinsic contractile state

A) 4 work loops were performed on each day of culture. The work loops had different set of 3EWK parameters to cover a spectrum of systolic loads, but the same work loops were performed on each day of culture and on all LMS irrespective of culture load. The acquired data were used to fit a modified $E_t$ model and predict the load-independent contractile state of cultured LMS. Notice that all isochrones are constrained on the x-axis at ~ 8.2 mm corresponding to the RL of a 9 mm LMS cultured at 110% stretch. Only 1/3 of isochrones are shown here for clarity. B) $E_t$ transient. Each data point of this transient corresponds to the slope of an isochrone shown in panel A. C) QR code of visual LabVIEW code that run the modified time-varying elastance (password: phd?).
3.2.10 Frank-Starling and force-% stretch linear regressions

At end point culture, LMS were assessed in their response to stretch *in situ* the Aurora at 1 Hz stimulation. All LMS were first put into an isometric mode of contraction via the LabVIEW software to control for the same systolic load. Then, the position of the 300C lever was adjusted using a digital knob on the front panel of the Aurora hardware box, which lengthened the LMS. LMS cultured under isometric load, physiological load and pressure-overload were stretched from RL to 126.3% whilst volume-overloaded LMS were stretched to 138.5%, in ~ 2% stretch steps, and kept for ~ 3 min in each % stretch. For the volume-overloaded LMS the higher terminal % stretch was chosen as preliminary work indicated that isometric tension did not peak at 126.3% of RL.

Analysis of force production at each % stretch was done using custom LabVIEW programs that automatically determined amplitude and baseline of the acquired force data (2.10.1.2). To assess the sensitivity of LMS to mechanical stretch a linear regression was fit to the force-% stretch data as described in 2.10.2 and the slopes of the lines compared.

3.2.11 Tissue viability

The viability of LMS cultured in the Aurora was determined using CellTiter 96 assay (Promega, UK), as described in (Watson *et al.*, 2019). LMS were removed from the Aurora by holding them from their rings using forceps and placed in a petri dish. The rings were dissected out of the LMS by using a stainless-steel blade, and a 2 mm diameter sample was obtained from the centre of the LMS using a sterile disposable biopsy puncher (VWR, UK). The sample was placed in a well of 96-well plate (BIO-RAD, UK) filled with 100 μl Media-199 + 40 μl of CellTiter 96 and incubated for 20 min at 37 ºC, 95% O₂ 5% CO₂ inside an incubator. Then, the sample was removed and the absorbance of the solution in the well measured at 490 nm wavelength using a plate reader (Labtech, USA). The absorbance of background of a separate well (containing incubated Media-199 + 40 μl CellTiter 96, no LMS) was also measured. The tissue viability was calculated by subtracting the background absorbance from sample absorbance.

3.2.12 pH of culture media bathing the LMS

The pH of the media bathing the LMS was determined on the final day of culture. After removing the LMS, the media was transferred to a sterile bottle, magnetically stirred, and measured using a calibrated pH probe (Mettler Toledo, UK).
3.2.13 Immunohistochemistry

LMS were stained with WGA as detailed in Table 2.4 and 2.8.2. Cardiomyocyte dimensions (area, width, length, aspect ratio) were then quantified by two blinded independent reviewers as outlined in 2.10.4.1 and 2.10.4.2, and shown in Figure 3.5.
Figure 3.5: Analysis of cardiomyocyte dimensions of 3-day cultured LMS using wheat germ agglutin (WGA)

8 cardiomyocytes were analysed per image and 5 images obtained per biological replicate. Analysis was done blinded. Figure adapted from (Pitoulis et al., 2021) under CCL
The protein expression of LMS cultured for 3 days in physiological, pressure-, or volume-overload was probed using western blots. LMS were quickly washed in sterile PBS, patted dry on a tissue roll, and snap frozen in liquid N\textsubscript{2}. They were then stored in -80 °C until further processing. The following protocol has also been described in (Pitoulis \textit{et al.}, 2021).

First, the protein content of the samples was determined. LMS were placed in 2 ml Eppendorf tubes (Eppendorf, UK) containing 2 mm diameter stainless-steel beads (Qiagen, UK) and 300 μl of RIPA buffer supplemented with protease and phosphatase inhibitors (10 ml of RIPA + 1 pill of each) (Thermofischer, UK). They were then mechanically digested for 4 min or until the Eppendorfs were optically clear by using TissueLyser LT (Qiagen, UK) at 4 °C. The Eppendorfs were then centrifuged for 20 min at 4 °C, 12000 rpm (Centrifuge 5804 R, Eppendorf, UK), the supernatant obtained using a 1000 μl sterile filtered pipette and kept in a sterile Eppendorf. 10 μl of the supernatant were obtained for determination of protein concentration using the Pierce BCA kit by comparing against a standard calibrated curve as detailed by the manufacturer’s instructions (Thermofischer, UK). The remainder were stored at -80 °C until further processing.

The samples were defrosted, vortexed, and 30 μg of protein calculated based on the protein concentrations were mixed with 5 μl of 4X Bolt LDS sample buffer (Thermofischer, UK), 5% 2-mercaptoethanol (VWR, UK) and dH\textsubscript{2}O for a total volume of 25 μl. After warming the samples at 70 °C for 10 min using a thermocycler (BIO-RAD, UK), they were loaded on a 17-well 4-12% SDS-PAGE gel previously filled with 20x MES SDS Bolt running buffer (Thermofischer, UK). Standard spectra multicolour broad range protein ladders (Thermofischer, UK) were loaded on wells 1 and 17, and the gel run for 25 min at 200 V (BIO-RAD, UK). While the gel was running, the transfer sandwich was prepared by activating 0.45 μM PVDF membranes in methanol and soaking the filter papers and sponge pads in transfer buffer (5% Bolt transfer buffer (Thermofischer, UK), 10% Ethanol, 1% 2-mercaptoethanol diluted in dH\textsubscript{2}O).

Bubbles were removed from the sandwich using a roller, and proteins transferred electrophoretically at 60 V for 20 min using the same electrophoretic cell. The PVDF membrane was then blocked using blocking solution (3% BSA diluted in 1X TBS-T (Thermofischer, UK)) for 1 h on a rocker. It was cut based on molecular weight of the proteins of interest and incubated with primary antibodies diluted in blocking solution at 4 °C on a rocker overnight. After incubation, the membranes were washed in TBS-T three times.
for 15 min on a rocker. They were incubated with secondary antibodies in TBS-T covered with aluminium foil on a rocker for 2 h, and then washed a final three times in TBS-T. The membranes were kept in 50 ml Falcon tubes (Corning UK) at 4 ºC and visualised within 1 day. Bands of interest where visualised using the ChemiDoc Touch Imaging system (BIO-RAD, UK). The primary and secondary antibodies used, and their respective dilutions are summarised in the table below (Table 3.1)

**Table 3.1: Primary and secondary antibodies used for western blots**

<table>
<thead>
<tr>
<th>1° antibody</th>
<th>Dilution</th>
<th>Molecular weight (kDa)</th>
<th>2° antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit GAPDH (Cell Signalling, UK)</td>
<td>1:1000</td>
<td>36</td>
<td>IRDye 680RD donkey anti-rabbit</td>
<td>LICOR Biosciences, UK</td>
</tr>
<tr>
<td>Rabbit type 1 collagen (Abcam, UK)</td>
<td>1:1000</td>
<td>138</td>
<td>IRDye 680RD donkey anti-rabbit</td>
<td></td>
</tr>
<tr>
<td>Mouse α-actinin (Abcam, UK)</td>
<td>1:1000</td>
<td>100</td>
<td>IRDye 800CW donkey anti-mouse</td>
<td>1:2000</td>
</tr>
<tr>
<td>Rabbit myosin heavy chain (Abcam, UK)</td>
<td>1:800</td>
<td>220</td>
<td>IRDye 680RD donkey anti-rabbit</td>
<td></td>
</tr>
<tr>
<td>Rabbit myosin light chain 2 (Abcam, UK)</td>
<td>1:1000</td>
<td>19</td>
<td>IRDye 680RD donkey anti-rabbit</td>
<td></td>
</tr>
</tbody>
</table>

The protein content of western blot gels was quantified using ImageJ. Raw composite images produced by the ChemiDoc imaging system were first converted to 8-bit, and the brightness of the whole image adjusted. This made visualisation easier but did not affect the results as all biological replicates were on the same gel. The bands of interest were selected using the ‘rectangular selection’ tool, and the lanes plotted using ImageJ’s built-in functionality. The ‘straight’ tool was then used to enclose bands and the ‘wand’ tool to measure the area of each. The % of total area of each band was measured and the data saved. This was repeated for all proteins. Results were normalised to GAPDH, apart from collagen.
type 1, which was not, as fibrosis was defined as the absolute amount of collagen in the LMS (Figure 3.6).
Figure 3.6: Analysis of western blot gels
A) Composite images were first converted to black/white by using an 8-bit transform, and the brightness adjusted. The lane was then selected, and the band profiles plotted. B) Plot profiles and results table.
### Transcriptomic profile of cultured LMS

The transcriptomic profile of LMS was investigated using RNA sequencing in collaboration with Dr Ke Xiao, Dr Saskia Mitzka, Dr Filippo Perbellini, and Prof Thomas Thum at Hannover Medical School, Institute for Molecular and Translational Therapeutic Strategies, Germany. The protocol has also been described in (Pitoulis et al., 2021).

#### 3.2.15.1 RNA Extraction

Processing of samples was done blinded. LMS were washed in PBS for 10 s, patted dry on tissue role, and snap frozen in liquid N\(_2\) and stored in -80 °C until further processing.

For the extraction of mRNA the samples were first mechanically disrupted. Frozen LMS were placed in 2 ml sterile vials with 2 mm ceramic beads (Bertin Instruments, FRA) and 700 μl of Qiazol (Qiagen, DE). The vials were then placed in a Precellys 24 (Bertin Instruments, FRA) tissue homogeniser, which was set to 5500 rpm for 20 s, twice. Then RNA was then purified with an miRNeasy Mini Kit and On-Column DNase Digestion by following the manual of the manufacture (both from Qiagen, DE). The vials containing the RNA were kept at −80 °C until next-generation sequencing.

#### 3.2.15.2 Next generation RNA sequencing

500 ng of RNA for each LMS were used for rRNA depletion procedure with ‘NEBNext® rRNA Depletion Kit (Human/Mouse/Rat), 96 rxns’ (E6310X; New England Biolabs, USA) followed by generation of a stranded cDNA library using ‘NEBNext® Ultra II Directional RNA Library Prep Kit for Illumina’ (E7760L; New England Biolabs, USA). An Illumina NextSeq 550 sequencer (Illumina, USA) with High Output Flowcell for 2 × 75 bp paired-end reads was then used for RNA sequencing. The sequencer outputted BCL files, which were exported in FASTQ format using bcl2fastq v2.20.0.422(Illumina, USA). The raw reads contained in the files were used for bioinformatic analysis.

#### 3.2.15.3 Functional annotation of next generation sequencing

The raw reads contained in the Illumina files were aligned to rat genome (rn6.0) with RNA STAR (Dobin et al., 2013). Only protein coding genes were used in the analysis and annotations obtained from iGenomes (Rattus_norvegicus/Ensembl/Rnor_6.0). The read counts from the raw files were normalised and gene differential expression then performed using DEseq2 with default settings (Love, Huber & Anders, 2014). Volume- and pressure-overloaded LMS were compared with physiologically overloaded LMS. Hierarchical clustering was applied to normalised counts using Cluster3 as described in (de Hoon et al.,...
Functional annotation and enrichment analysis of significantly regulated genes was performed in DAVID v6.8 with integrated GO database comparing volume-overloaded with. The minimum number of genes to compare was set to 2 for each corresponding pathway and the EASE score (p-value of Fisher Exact test) set to 0.05 for a significant match.

**3.2.6 Statistics**

Statistical analysis was performed in Prism8 as described in 2.13. P<0.05 was considered statistically significant and the following symbols were used for group comparisons:

- *, **, ***, ****: p<0.05, p<0.01, p<0.001, and p<0.0001 for physiological load vs. isometric load.
- *, **, ***, ****: p<0.05, p<0.01, p<0.001, and p<0.0001 for physiological load vs. volume-overload.
- ^, ^^, ^^^, ^^^^^: p<0.05, p<0.01, p<0.001, and p<0.0001 for physiological load vs. pressure-overload.
- !, !!, !!!, !!!!!!: p<0.05, p<0.01, p<0.001, and p<0.0001 for volume-overload vs. pressure-overload.
3.3 Results

3.3.1 SL-% stretch relationship

Laser diffraction experiments were performed on fresh LMS, and a linear regression fit to the data to quantify the % stretch required to reach a desired SL (SL-% stretch relationship). This enabled us to mechanically load LMS to any given SL, and thus preload, based their RL and the experimentally derived equation below, also shown in Figure 3.7.

\[ y = 0.01262x + 1.968 \quad (13) \]

Where \( y \) is SL, and \( x \) is % stretch.

\[ R^2 = 0.55. \]
Figure 3.7 Quantification of SL-% stretch relationship using laser diffraction experiments

A) The line equation was determined from laser diffraction and subsequently used to stretch LMS to the desired preload. 2.1 μm SL, corresponding to 110 % stretch, was used for isometric load, physiological load, and pressure-overload, while 2.3 μm SL, corresponding to 126 % stretch, was used for volume-overloaded LMS. B) Residual plot analysis of the data presented in (A). N = 18 LMS/6 biological replicates. (A) adapted from (Pitoulis et al., 2021) under CCL.


3.3.2 Optimisation of LMS culture settings

Although the focus of the project was the development of advanced electromechanical stimulation protocols a series of optimisations, deemed necessary for long-term LMS culture, were first performed.

3.3.2.1 Culture media

*In vivo* the heart is constantly exposed to pulsatile and steady levels of hormones including corticosteroids (cortisol) from the adrenal cortex, thyroid hormones (T3, T4) from the thyroid gland, and catecholamines (adrenaline, noradrenaline) from the adrenal medulla and cardiac sympathetic fibers. These fluctuate in response to the energetic demands of different tissues, increasing contractility, HR, and CO.

Past studies have shown that LMS cultured for 1 week on transwells without catecholamines develop hypersensitivity to β-AR stimulation (Brandenburger *et al.*, 2012). Likewise, RNAseq data shows that LMS cultured for 24 hr on fixed posts without catecholamines have dysregulated β-AR pathway (Watson *et al.*, 2019). Although abnormally high levels of such agents (e.g. sympathetic overdrive in HF) can cause aberrant myocardial remodelling and contractile deterioration, clinical trials such as the MOXCON and BEST (Cohn *et al.*, 2003)(Bristow *et al.*, 2004) have also shown that when their plasma levels are extremely low, adverse outcomes are favoured.

As the standard culture media does not contain hormones, basal levels were included in it, to mimic the *in vivo* tonic neurohormonal drive. Their concentration was based on a literature search and/or dose-response experiments on LMS (Figure 3.8).
Figure 3.8: Noradrenaline and triiodothyronine (T₃) acute dose-response curves

A) Noradrenaline dose-response curve on freshly prepared LMS at 2.2 μm SL. Data points are mean ± SEM; N = 9 LMS/3 biological replicates. B) T₃ dose-response curve on freshly prepared LMS stretched to 2.2 μm SL. N = 6 LMS/3 biological replicates. T₃ experiments were conducted by Dr Raquel Nunez-Toldra. Data points are mean ± SEM.
The noradrenaline dose-response curves were conducted across a range of concentrations either way of the physiological concentration in the rat plasma (equal to 2.5 nM) according to the literature (Popper, Chiueh & Kopin, 1977)(Buhler et al., 1978)(De Boer & Van Der Gugten, 1987). We found that at 2.5 nM noradrenaline, twitch force production of freshly prepared LMS was significantly increased compared to control (p<0.05) (Figure 3.8). As we intended to mimic a tonic adrenergic state, a noradrenaline concentration of 2.5 nM was used to supplement the culture media. Adrenaline was also added to the media, and its concentration set to 4 nM based on literature values (Table 3.2). The culture media was further supplemented with triiodothyronine (T₃). T₃ is an essential hormone for cardiac development and cardiac performance (Lee et al., 2010). In the perinatal period it promotes myosin heavy chain and titin fetal to adult isoform switch (Krüger et al., 2008) as well as the development of hypertrophy (Dillmann, 2010). It can have sympathomimetic actions acutely (Hoit et al., 1997), while long-term effects include an increase in numbers and sensitivity of β-adrenergic receptors (Williams et al., 1977)(Hoit et al., 1997). Given its physiological importance, dose-response experiments were conducted to identify the response of LMS on different doses of T₃. Like with noradrenaline, the results of guided us to choose the concentration of T₃ for culture (Figure 3.8). Our literature analysis showed that the rat plasma basal level of T₃ is ~ 2.15 nM (Waner & Nyska, 1988) (Table 3.2). We found that LMS contractility was significantly increased at 2.15 nM (p<0.05) compared to control, and as such this concentration was chosen to supplement the media. The addition of glucocorticoids in culture promotes maturation of hiPSC-CMs (Parikh et al., 2017), while absence of the glucocorticoid receptor in the mice leads to aberrant Ca²⁺ cycling and deterioration of contractile function (Oakley et al., 2013). Although preparation of LMS using the vibratome results in minimal damage compared to other techniques (e.g. enzymatic isolation for single cells), multiple inflammatory markers are upregulated, and cardiac enzyme have been shown to be released (Parrish et al., 1992). We hypothesized that addition of corticosteroids at basal plasma levels could ameliorate these effects. Basal rat plasma levels of dexamethasone were added during LMS culture. The concentration of these were determined from literature values (Table 3.2).
Table 3.2: Hormonal supplementation of culture media

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Basal rat plasma concentration</th>
<th>Concentration used in culture</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)-Adrenaline (+)-bitartrate salt</td>
<td>~ 4 nM</td>
<td>4 nM</td>
<td>(Popper, Chiueh &amp; Kopin, 1977), (Buhler et al., 1978)</td>
</tr>
<tr>
<td>L-(-)-Noradrenaline (+)-bitartrate salt</td>
<td>~ 2.5 nM</td>
<td>2.5^1</td>
<td>(Popper, Chiueh &amp; Kopin, 1977), (Buhler et al., 1978)</td>
</tr>
<tr>
<td>monohydrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3,3',5-Triiodo-L-thyronine</td>
<td>~ 2.15 nM</td>
<td>2.15^1</td>
<td>(Waner &amp; Nyska, 1988)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>~ 100 nM</td>
<td>100</td>
<td>(Atkinson &amp; Waddell, 1997)</td>
</tr>
</tbody>
</table>

3.3.2.2 Rings

3D printed rings were used throughout the project to physically manipulate the LMS. An array of different designs of these were developed. The first were triangular shaped as shown in the video of Figure 3.3 and top left panel of Figure 3.9. However, these were difficult to manipulate using forceps. Rectangular shaped rings were thus designed and printed next. These were used for acute LMS experiments, including the experiments described in Chapter 5, and dose-response curves 3.3.2.1 described here. Although manipulation of these was easier, they were impractical for culture, where LMS are suspended from two opposite stainless-steel needles. Specifically, as the LMS were continuously shortened/stretched during culture, lack of a secure fit often caused the needles to slip against the rings, unhooking and unloading the LMS. Given that mechanical load was the independent variable in the culture experiments, this was a major confounding problem. To solve it, we designed and printed rectangular rings with a slit halfway across their length. These permitted the needles to be secured in place preventing unwanted slippage. These are shown in the video of Figure 3.10. For MyoLoop a different ring design was used, as shown on the bottom right panel of Figure 3.9. The rationale for this is described in Chapter 4.

^1 Experimentally determined.
Figure 3.9: Renderings of different ring designs used with LMS
A) Triangular rings. B) Rectangular rings. These were primarily used for acute experiments. C) Rectangular rings with single central slit. These were used to keep the LMS secure during *in vitro* culture in the Aurora by mounting them between the opposite posts. D) Rectangular rings with multi-positional slits. These were used for culture of LMS in MyoLoop. All rings were printed in Ultimaker 3 Cura using PLA filament as detailed in 2.5.
We set out to address objective (a) and investigate whether chronic physiological mechanical load using a 3EWK model would be 1) tolerated by LMS, and 2) superior to an isometric culture protocol, the lab’s standard methodology at the time.

LMS were cultured at 2.1 μm SL under isometric load or physiological afterload using a 3EWK model (Ra = 0.7, Rc = 0.5, Ca = 0.5) (Figure 3.10). Given that we only had one Aurora set-up, these early validation experiments were limited to 24 hr. The short timescale permitted rapid data acquisition of an untested low-throughput system together with rapid optimisation of culture parameters, such as the ring design, culture media, and time-varying elastance algorithms.

Irrespective of mechanical load applied, LMS showed a biphasic response in their developed force over the 24 hr culture, characterised by an initial run-down in force development during the first hours and a steep increase after ~ 10 h (Figure 3.11). This is in contrast to past experiments from our lab on rabbit LMS, which when cultured under isometric load show steady force production throughout culture (Watson et al., 2019).

However, there are numerous differences between the experiments here and those described by (Watson et al., 2019) including different species, different method of animal sacrifice, different culture media, different preload (2.1 vs. 2.2 μm) and different culture set-up. Others have found that when human LMS are cultured in auxotonic load, there is an initial large drop followed by progressive increase in contractility (Fischer et al., 2019). As the U-shaped response reported here was common to both isometric and physiological load, we concluded that it was a shared response to the in vitro culture environment and/or method of preparation of LMS and not related to load. This was also bolstered by the subsequent longer (3 day) experiments on physiological load, pressure-, or volume-overload, which showed the same initial drop in LMS force development followed by upswing irrespective of load (Figure 3.13E).

$E_{\text{max}}$, assessed after 24 hr culture, was significantly decreased in isometric load compared to physiological load, meaning that the contractile capacity of the former was compromised after culture (Figure 3.11). This was also supported by the Frank-Starling results. Although physiologically loaded LMS had a positive force-stretch response, isometrically loaded LMS had an initial increase followed by a descending arm and were significantly weaker and stiffer at the highest % stretch (126%, p<0.001) (Figure 3.11).
These experiments demonstrated that the application of dynamic mechanical load, in the
form of a working cardiac cycle, is not only well tolerated by the LMS but also advantageous
to the preservation of contractile LMS properties. Our next objective was to compare
pathophysiologically relevant load profiles over a greater culture duration.
Figure 3.10: Representative traces of isometric or physiological load at 2.1 μm SL
A) Representative work loops of LMS cultured for 24 hr in the Aurora under isometric or physiological load. LMS were stretched to the same preload (2.1 μm) at the beginning of culture and either isometric or physiological afterload. B) QR code of LMS beating under isometric or physiological load at the beginning of culture (password: phd?).
Figure 3.11: Contractile function of LMS cultured under isometric or physiological load during and after 24 hr

A) Force developed by LMS during in vitro culture. Notice U-shaped response (N = 5, 4 for isometric and physiological load respectively). B) $E_{\text{max}}$ of LMS after 24 hr culture in isometric or physiological load (N = 4). C, D) Active and Passive Frank-Starling relationship of LMS cultured for 24 hr in isometric or physiological load (N = 4). Data shown as mean ± SEM.
3.3.4 LMS culture under physiological, pressure-, or volume-overload

Despite the encouraging 24 hr data, the electromechanical culture was compromised by its short duration and the unphysiological nature of isometric load. We increased culture duration to 3 days, and exposed LMS to physiological load, pressure-overload, or volume-overload, pathophysiologically relevant mechanical load allowing us to address objective (b). To do that, the afterload of all LMS was imposed using the 3EWK model while the preload was set by stretching LMS to the desired SL. For physiological load, LMS were cultured under normal preload and afterload, for pressure-overload under normal preload but high afterload, and for volume-overload under high preload and normal afterload. The exact parameters and rationale for these are described below and summarised in Table 3.3 and the work loops performed by LMS immediately once put on the Aurora are shown in Figure 3.12.

3.3.4.1 Rationale for chosen parameters of preload and afterload

2.1 μm SL was chosen as normal preload and was used to culture of physiologically loaded and pressure-overloaded LMS. 2.3 μm was chosen as high preload and was used to for the volume-overloaded LMS.

The first motivation for the chosen SLs were previous data acquired in our lab by (Watson et al., 2019), who cultured LMS for 24 hr at 2.4 μm SL and showed aberrant electromechanical, metabolic, structural, and transcriptomic remodelling. As the experiments conducted in this thesis aimed to prolong the culture to 3 days, it was decided that a preload of 2.4 μm may be too disruptive; hence 2.3 μm SL was used to simulate volume-overload. For physiological load and pressure-overload, 2.1 μm SL was chosen as it reflected an intermediate value of the SL measured in vivo, which varies between ~1.9 to 2.2 μm during the cardiac cycle in the dog (Rodriguez et al., 2017). The second motivation behind the SL values were literature data for LV diameter or volume. The LV diameter of volume-overloaded rats is increased by ~30%, close to the 126% RL stretch value chosen here for volume-overloaded LMS. Likewise, if a spherical model of the LV is used to convert LV volume to radius, an ~26% increase in radius can be calculated from Figure 1 of (Toischer et al., 2010) for volume-overloaded (aortocaval shunt) mice which show that their LV volume increases from 15 μl to 30 μl (see equation(14)).
\[ V = \frac{4}{3} \times \pi r^3 \]
\[ r_{30 \, \mu m} = \left( \frac{3 \times 30}{4\pi} \right)^{\frac{1}{3}} = 1.26 \]
\[ r_{15 \, \mu m} = \left( \frac{3 \times 15}{4\pi} \right)^{\frac{1}{3}} = \]

It is important to note that although the SLs chosen are supported by the literature to correspond to normal or pathological preload, given the paucity of *in vivo* data for SL, interpretation of the SL values should be made relatively to one another, that is 2.1 μm being normal vs. 2.3 μm being indicative of higher preload.

Afterload was imposed by controlling the LMS shortening rate and amount (stroke length) during the systolic phase using the 3EWK model. The predictions depended on the input force-transient, as well as the Ra, Rc, and Ca components of the model. The exact numerical values for Ra, Rc, and Ca, were chosen based on their ratios which have been suggested to be in the physiological range when Rc/Ra = 0.07-0.09 (Westerhof & Elzinga, 1991). Thus, these were set to Ra = 0.7, Rc = 0.5, and Ca = 0.5 for physiological and volume-overload, whereas for pressure-overload Ra = 10, Rc = 0.9, and Ca = 0.3 were chosen, simulating increased aortic impedance and peripheral resistance, and decreased aortic compliance. From hypertensive data acquired *in vivo*, Ra and Rc have been shown to increase by a factor of ~1.45 whereas Ca decreases by a factor of ~0.46 (Kind *et al.*, 2010). Here, in pressure-overload, Ra increased by 1.43, Rc by 1.8, and Ca decreased by 0.6. Ultimately, and akin to the preload values, the 3EWK afterload parameters should be interpreted relative to each other – that is, high afterload in pressure-overload vs. normal afterload in physiological load and volume overload.

**Table 3.3 Mechanical load parameters used to culture LMS *in vitro***

<table>
<thead>
<tr>
<th>Group</th>
<th>Preload</th>
<th>SL (μm) / % stretch</th>
<th>Afterload</th>
<th>3EWK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ra</td>
</tr>
<tr>
<td>Physiological load</td>
<td>←→</td>
<td>2.1 μm / 110</td>
<td>←→</td>
<td>7</td>
</tr>
<tr>
<td>Pressure-overload</td>
<td>←→</td>
<td>2.1 μm / 110</td>
<td>↑</td>
<td>10</td>
</tr>
<tr>
<td>Volume-overload</td>
<td>↑</td>
<td>2.3 μm / 126</td>
<td>←→</td>
<td>7</td>
</tr>
</tbody>
</table>
Figure 3.12: Representative traces of LMS cultured under physiological load, pressure-overload, or volume-overload

A) Work loops of LMS cultured under the different load profiles. These are representative loops performed by the LMS at the beginning of culture (day 0, hour 0). B) Force and length transients of the work loops shown in (A). Note that the diastolic phase was the same for all groups but was not acquired by the Aurora and thus loops appear open. Figure adapted from (Pitoulis et al., 2021) under CCL.
3.3.4.2 State of the art electromechanical culture with dynamic afterload

The overarching goal of the 3 day culture was to study the effects of load on myocardial phenotype. A design feature of our methodology briefly described in 3.2.7 was essential to do that robustly. It is detailed here in full.

A remodelling preparation is constantly adapting to its environment, undergoing changes in its electrophysiology, metabolism, and contractility (Pitoulis & Terracciano, 2020). Within the setting of an LMS beating in the Aurora, these changes were detected in real time by sampling the force-transient generated by the LMS. These force-transients were then fed to the 3EWK which outputted the length transients that were subsequently applied to the LMS. Thus, a change in the dependent variable (force) triggered a change in the independent variable (length) continuously and throughout culture: a close-feedback loop. This is necessary for physiological relevance to be captured.

Previously we (Pitoulis et al., 2019a), and more recently others (Ng et al., 2020) have performed work loops in vitro on cardiac tissue by application of precalculated length transients. Under such a framework, the tissue is always shortening with the same rate and stroke length irrespective of its force production characteristics, which means that afterload is not being controlled for. Here, by using an iterative PID closed-feedback loop approach to apply length-transients based on force-transients, the rate and extent of shortening change based on what the LMS is doing at any given time, controlling for afterload during culture. For example, for the same afterload (same 3EWK parameters), the volume-overloaded LMS shorten more than physiologically loaded LMS on day 0 of culture, as they generate more force. However, as the LMS in each load group remodel during culture and force output changes, the length transients predicted and actuated by the Aurora change in sync to mirror that (Figure 3.13B-C).

As the Aurora constantly sampled force and length data during culture, contractile remodelling was temporally resolved. On day 0, volume-overloaded LMS had the greatest stroke length, followed by physiologically loaded, and then pressure-overloaded LMS. This follows the fact that the same afterload was imposed on volume- and physiologically loaded LMS, but the former were at a higher preload, producing more force according to Frank-Starling and thus shortening more. Likewise, as a higher afterload was applied to pressure-overloaded LMS at the same preload as physiologically loaded LMS (Table 3.3), these shortened the least. On the subsequent days of culture volume-overloaded LMS showed a monotonic decrease in force generation mirrored by a decreasing stroke length (Figure
In contrast, physiologically loaded and pressure-overloaded LMS increased their force production, stabilising at day 3.
Figure 3.13: Temporal load-induced contractile remodelling

A) Closed-feedback method to iteratively load LMS during culture. B, C) Representative work loops and force-length transients across culture. D) $E_{\text{max}}$, corresponding to load-independent intrinsic contractile state. Note: all LMS start at the same $E_{\text{max}}$. E) LMS twitch force throughout culture. N = 6. Data shown as mean ± SEM. Adapted from (Pitoulis et al., 2021) under CCL.
3.3.4.3 Time varying elastance ($E_t$) to assess LMS load-independent contractility

The protocol and algorithms used to assess time-varying elastance have been described in 3.2.9. Here we detail the rationale and the results of these experiments.

Because LMS from different groups were beating against different preload and/or afterload during the 3-day culture, twitch force which depends on diastolic and systolic load could not be used reliably to quantify contractility. For example, on day 0, volume-overloaded LMS produced significantly greater twitch force than physiologically loaded LMS (Figure 3.13E). This does not reflect stronger tissue, but rather different loading conditions. Specifically, the volume-overloaded LMS being at the same afterload (same 3EWK parameters) but higher preload than physiologically loaded LMS (2.3 vs 2.1 μm SL), which results in greater force development according to length-dependent activation and Frank-Starling mechanics (Allen & Kentish, 1985).

We modified the classical $E_t$ theory to obtain a load-independent measure of intrinsic LMS contractility, $E_{\text{max}}$, which considers both the length and the force of a series of work loops (Figure 3.4) and that is also used clinically from PV loop analysis (Sagawa, 1981). In support of this method, although the twitch force developed by LMS in the different groups on day 0 was different (Figure 3.13B, E), the $E_{\text{max}}$ was not (Figure 3.13D). This is sensible as LMS had not been yet subjected to the remodelling effects of chronic culture.

Volume-overloaded LMS had significantly decreased $E_{\text{max}}$ at day 1–3 compared to physiological loaded and day 3 compared to pressure-overloaded LMS (Figure 3.13D). This is appreciated on the work loop phase plane by loops of decreasing height and width (Figure 3.13B-C). In contrast, pressure-overloaded LMS showed preserved $E_{\text{max}}$ throughout culture. Together these findings propose that, within the timeframe and amount of overload examined here, pathological preload is more damaging to contractile performance than pathological afterload.

Interestingly, the $E_{\text{max}}$ of physiologically loaded LMS increased at day 2 compared to day 0 (Figure 3.13D). Although it is unclear why this happens, LMS undergo an initial ‘acclimatisation’ phase, characterised by a U-shaped response with initial rapid force decline followed by an upswing (Figure 3.11A & Figure 3.13E). This, most likely a consequence of method of LMS preparation (low Ca$^{2+}$: K$^+$ ratio, high 2,3-BDM, Tyrode’s at 4 ºC) and first exposure to the artificiality of culture environment, may hamper force production equivalently for all groups at the beginning of culture. Subsequently, during the upswing, the
less disruptive physiological load may allow LMS to attain greater force than that afforded by pathological load.

3.3.4.4 Frank-Starling relationships of cultured LMS

The Frank-Starling response is a fundamental concept in cardiac physiology. At the level of the whole heart, it relates an increase in venous return (preload) to an increase in contractility (and by extension cardiac output) and when disrupted, the ability of the myocardium to respond to stress is compromised (Pitoulis & de Tombe, 2019). We performed isometric Frank-Starling experiments to elucidate the effect of chronic differential mechanical load on the ability of LMS to respond to mechanical stress.

The culture preload (2.1 or 2.3 μm) was applied to LMS at the beginning of culture based on the SL-% stretch relationship obtained from the laser diffraction experiments. However, *in vitro* culture can induce changes in SL (Yu & Russell, 2005)(Mansour *et al.*, 2004), and these are known to occur in the presence of pressure- or volume-overload *in vivo* (Kehat & Molkentin, 2010)(Grossman, Jones & McLaurin, 1975). Thus, as SL could have changed during culture and differently for each group, it could not be used reliably as the independent variable; instead % stretch from RL was used and the results of the Frank-Starling experiments reported as such.

All LMS showed a positive Frank-Starling response increasing force output per unit stretch, however; each group had a unique trajectory. The pressure-overloaded LMS followed a similar response to the physiologically loaded LMS, however their max force output at the highest % stretches (116-126 %) was significantly decreased (Figure 3.14B). Additionally, the slope of the force-stretch response was significantly lower, suggesting decreased sensitivity to stretch (Figure 3.14D). In view of the preserved $E_{\text{max}}$ (Figure 3.13) these results suggest that pressure-overloaded LMS can produce force at baseline but have an inadequate response to mechanical stress. In contrast, volume-overloaded LMS had a rightward shift in their Frank-Starling response with force production significantly diminished across the entirety of the stretch spectrum investigated. As these LMS also showed decreased $E_{\text{max}}$, these results suggest greater contractile depression and systolic dysfunction. Although the passive force of both pressure-, and volume-overloaded LMS was significantly decreased (i.e. increased $\Delta \text{stretch}/\Delta \text{Force}$), it was disrupted more in volume-overloaded LMS(Figure 3.14C), suggesting alterations in the passive mechanical properties similar to those observed in dilating phenotypes (Opie *et al.*, 2006).
Finally, lack of differences in LMS viability means that these functional findings were not underscored by tissue death (Figure 3.14A) but were instead caused by aberrant contractile remodelling due to the different mechanical profiles imposed on them.
Figure 3.14: Viability and contractile remodelling of 3 day cultured LMS
A) Tissue viability of cultured LMS (N = 5). B, C) Active and Passive Frank-Starling relationships of cultured LMS (N = 6, 6, 4 for physiological load, volume-overload, and pressure-overload respectively). D) Active force - % stretch slopes derived from (B). Data shown as mean ± SEM. Figures adapted from (Pitoulis et al., 2021) under CCL.
3.3.4.5 Cardiomyocyte area, width, and length of cultured LMS

To determine the effect of mechanical load on cardiomyocyte dimensions, LMS were fixed, stained with WGA, confocal images acquired and the data analysed by two blinded reviewers as described in 2.8, 2.10.4, 3.2.13, and shown in Figure 3.5. Pressure-overload significantly increased cardiomyocyte area and length after 3 days in culture compared to physiological load (Figure 3.15). There were no significant differences in area, width, or length between physiological load and volume-overload.
Figure 3.15: Cardiomyocyte dimensions of cultured LMS
A) Representative images of WGA staining at 20X magnification. B, C, D) Cardiomyocyte area, length, and width. Data as median ± IQR for B, C and mean ± SEM for D (N = 120/3, 159/4, and 184/5 for physiological, pressure-, and volume-overload). Figures adapted from (Pitoulis et al., 2021) under CCL.
3.3.4.6 RNAseq of cultured LMS

Pressure- and volume-overload have been shown to induce the expression of different genes in vivo (Toischer et al., 2010). To identify shared and load-specific expression signatures, the gene profile of cultured LMS was probed using RNAseq and analysed blinded by Dr Ke Xiao as described in 3.2.15.

The expression of 442 and 353 protein coding genes was significantly different in pressure-overloaded and volume-overloaded LMS compared to physiological load and 62 of these genes were shared in both groups (Figure 3.16). Therefore, there were 380 and 291 genes that were unique to either condition. In volume-overloaded LMS, gene ontology analysis detected pathways involved in heart development (GO:0055013, 0055001, 0055006, 0055002), and assembly and organisation of the sarcomere apparatus (GO:0030239, 0031032) (Figure 3.17). Furthermore, at least 13 genes of the PI3K-mTOR pathway, a mediator of eccentric remodelling in response to increased diastolic wall stress (Ikeda et al., 2015) were upregulated in volume-overloaded LMS (Table 3.4). These genes were not picked up in pathway analysis of pressure-overloaded LMS, which in contrast showed predominantly dysregulation of inflammatory and stress pathways (GO:0050794) including multiple genes downstream of NF-κβ, as well as genes involved in metabolism (GO:0080090, GO: 0031323) (Figure 3.17, and Table 3.4).

61/62 genes shared between pressure- and volume-overload showed the same direction of expression (i.e. upregulated or downregulated in both); these are highlighted in turquoise colour in Figure 3.16. Actn1 was the only gene out of these that showed opposite direction of expression, upregulated in volume-overloaded but downregulated in pressure-overloaded LMS and shown in pink in Figure 3.16.

To validate the gene expression findings, we performed western blots for a number of these genes. The gene expression of myosin light chain 2 (MYL2), a contractile protein found in the sarcomeres, was significantly downregulated in volume-overloaded but not significantly different in pressure-overloaded LMS compared to physiological load. Protein levels of MYL2 were significantly decreased in both overloaded groups (Figure 3.16). There were no significant differences in the protein levels of Actn1, type 1 collagen, myosin heavy chain, despite the differences in gene expression (Figure 3.16).
Figure 3.16: Gene and protein expression of cultured LMS
A) Gene Venn diagram. B) Heatmap of shared genes expression. C) Volcano plots. D) Protein expression. E) Blots. N = 4, 5 for gene and protein expression. Data shown as mean ± SEM. Adapted from (Pitoulis et al., 2021) under CCL.
Table 3.4: Expression of selected genes of pressure- and volume-overloaded LMS compared to physiological load

<table>
<thead>
<tr>
<th>Gene</th>
<th>ΔVO</th>
<th>ΔPO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellular Adhesion Molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Itga3</td>
<td>↑</td>
<td>n.s</td>
</tr>
<tr>
<td>Itga9</td>
<td>↓</td>
<td>n.s</td>
</tr>
<tr>
<td>Itgbl1</td>
<td>↑</td>
<td>n.s</td>
</tr>
<tr>
<td>rhoc</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>ankrd37</td>
<td>n.s</td>
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</tr>
<tr>
<td>ankrd52</td>
<td>n.s</td>
<td>↑</td>
</tr>
<tr>
<td><strong>PI3K-Akt</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Igf</td>
<td>↑</td>
<td>n.s</td>
</tr>
<tr>
<td>Fgfr2</td>
<td>↑</td>
<td>n.s</td>
</tr>
<tr>
<td>Ngfr</td>
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<td>n.s</td>
</tr>
<tr>
<td>Vegfa</td>
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<td>n.s</td>
</tr>
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Figure 3.17: Functional enrichment analysis for pathway identification in cultured LMS
A, B) Gene ontology analysis for pressure-, and volume-overloaded LMS respectively (N = 4). Figures adapted from (Pitoulis et al., 2021) under CCL.
3.4 Discussion

In this chapter we aimed to a) advance electromechanical LMS culture by using a physiologically relevant mechanical loading approach, and b) study load-induced remodelling in vitro. We found that LMS functional phenotype is preserved better using cardiac work loops than isometric load. Using our platform and culture methodology we show that we can recreate load-specific myocardial phenotypes in vitro.

3.4.1 Advanced electromechanical LMS culture

In our lab, the standard methodology to culture LMS in vitro has been to use stretchers with inflexible posts, whereby LMS are stretched to the desired diastolic length and then beat under in an isometric mode of contraction (Figure 1.9). The 24 hr functional data of physiological vs. isometric load, suggested that although viable, LMS cultured with isometric load showed signs of aberrant contractile remodelling including diminished $E_{\text{max}}$, and Frank-Starling response. The mechanistic basis of these results was not explored, yet a few conclusions can be drawn from first principles.

Isometric contractions never occur in vivo. An isometric beat means that there is no tissue shortening as afterload is higher than what the tissue can pull (Sonneblick & Downing, 1963). Physiologically, an isometric contraction consumes the greatest amount of oxygen per beat (Suga, Hayashi & Shirahata, 1981) and may thus put the LMS at an unsustainable metabolic state. Furthermore, the mechanics of an isometric beat are not only deleterious during systole, but also for diastole. The absence of a shortening phase during systole means that the tissue is not being stretched back during diastole, and thus the cyclic pattern of shortening/re-stretching is disrupted. This state, where ventricular filling is compromised, represents a state of diastolic dysfunction (Aggarwal, Brown & LeWinter, 2001), and may explain why the passive force Frank-Starling curves were significantly stiffer in the isometrically cultured LMS (Figure 3.11C).

3.4.2 Hypertrophy and structural LMS remodelling as a function of mechanical load

One of the generally accepted tenets of remodelling is the cardiac hypertrophic response to mechanical overload. According to Laplace’s law (Grossman & Paulus, 2013) this occurs to normalise systolic stress as described in 1.1.2 and according to equation (3).

We found that mechanical overload induced cardiomyocyte hypertrophy with an increase in length and area in LMS cultured under pressure-overload for 3 days compared to physiological load.
Traditionally, pressure- and volume-overload are classified based on the ventricular hypertrophy pattern they cause into concentric and eccentric respectively (Pitoulis & Terracciano, 2020). However, evidence suggests that overlap may exist between the two and binary classification may be too crude (Opie et al., 2006). For example, cardiomyocytes from aortic stenosis patients, a condition of pressure-overload, show both increased length and width (Antonini-Canterin et al., 2003)(Villari et al., 1995)(Krayenbuehl et al., 1989).

**In vivo**, volume-overload induces increases in both the LV internal diameter and thickness (Mihl, Dassen & Kuipers, 2008), and at least in the compensated state the latter is essential for the greater amount of blood filling the heart to be ejected (Toischer et al., 2010).

Although cardiomyocytes from volume-overloaded LMS exhibited a trend of increased area, and length this did not reach statistical significance.

### 3.4.3 Mechanical overload induced contractile remodelling

The contractile performance of both overloaded groups was impaired. \(E_{\text{max}}\) was decreased and the Frank-Starling relationship shifted to the right in volume-overloaded LMS, akin to cardiac performance seen in overt systolic dysfunction (Sagawa, 1981)(Walley, 2016). In pressure-overloaded LMS, \(E_{\text{max}}\) was preserved but the ability to increase force output upon stretch was compromised and similar force-stretch trajectories have been shown in the pressure-overloaded cat RV **in vivo** (Spann et al., 1967), and **ex vivo** hearts of spontaneously hypertensive rats (Hallbäck, Isaksson & Noresson, 1975).

Whether pathological preload or afterload is more deleterious to cardiac function has not been conclusive. In a comparison of chronic mitral regurgitation, a state of volume-overload, and chronic aortic stenosis, a state of pressure-overload, in dogs, contractile function deteriorates more in the former (Carabello et al., 1992). Mechanistically, this has been suggested to be due to greater hypertrophic response in pressure-overload, which may thus normalise the excess haemodynamic overload (Carabello et al., 1992). Likewise, we reported that the hypertrophic response was greater in pressure-overloaded LMS, and the contractile dysfunction worse in volume-overloaded LMS. However, others have reported that pressure-overload induced by transthoracic aortic constriction (TAC) in mice is more damaging to the cardiac molecular and functional profile than volume-overload induced by aorto-caval shunt (Toischer et al., 2010). Yet, the shunt model employed by (Toischer et al., 2010) has been criticised as inducing ventricular unloading and not volume-overload. Specifically, (Reil, Hohl & Böhm, 2011) elegantly showed that the effective arterial elastance, a measure the total pulsatile load the heart is exposed to, and calculatable from the representative traces of
1969 Figure 1 of (Toischer et al., 2010), was significantly less in shunting (5.1 mmHg/μl) than
1970 TAC (28 mmHg/μl).

3.4.4 Pathophysiological consequences of a diminished contractile phenotype
1971 Volume-overloaded LMS had both an absolute decrease in force production across the %
1972 stretch range examined and a decrease in the slope of this response, which means that:
1973 I. A supraphysiological amount of stretch is required for meaningful force production
1974 (e.g. volume overloaded LMS needed to be stretched to ~130% stretch to produce
1975 the same amount of force as physiologically loaded LMS at ~ 110% stretch)
1976 II. The working range that the muscle is responsive to stretch is critically diminished
1978 A drop in CO in vivo is met with activation of a series of neuronal and hormonal
1979 regulatory reflexes (Triposkiadis et al., 2009), as well as shifting of fluid from the interstitial
1980 space to the vascular compartment, a consequence of the decrease in capillary hydrostatic
1981 pressure as dictated by basic Starling fluid forces (Miller, 2016). In concert, these responses
1982 increase contractility, effective circulating volume, and diastolic wall stress, pushing the heart
1983 to the right of the force-stretch response. At these higher stretches, as shown by our data, the
1984 myocardium would still be responsive to stretch and produce enough force to eject the excess
1985 blood. However, these compensatory mechanisms may be chronically unsustainable and,
1986 with recurring and continuous cycles of decompensation (Miller, 2016) lead to both
1987 decreased absolute force production at any given stretch and a smaller sensitive stretch range.
1988 This conceptual framework is supported by data from papillary muscles of terminal HF,
1989 which lose their Frank-Starling response (Schwinger et al., 1994).
1990 Furthermore, volume-overloaded LMS showed increased compliance based on their
1991 passive force-stretch curves, meaning that resistance to ventricular filling is decreased. This
1992 may be an intrinsic myocardial mechanism, which permits the heart to reach a higher
1993 operating preload and working in conjunction with the systemic compensatory mechanisms
1994 that raise intravascular volume. Although the mechanisms behind this were not explored
1995 hearts from DCM (Nagueh et al., 2004) and volume-overloaded rats (Virgen-Ortiz et al.,
1996 2009) show similar decreases in passive force with alterations in titin expression and isoform
1997 composition mechanistically implicated (Nagueh et al., 2004).

3.4.5 Differential load-induced gene expression
1999 In mice with volume-overload induced by arteriovenous fistula, eccentric remodelling
2000 involves activation of the phosphoinositide-3 kinase/protein kinase B/mammalian target of
rapamycin (PI3K/Akt/mTOR) signalling cascade (Ikeda et al., 2015)(Maillet, Van Berlo & Molkentin, 2013), and the activity of mTOR has been shown to increase in a diastolic wall stress-dependent manner (Ikeda et al., 2015). The PI3K pathway was upregulated in volume-overloaded but not pressure-overloaded LMS (Table 3.4). In contrast, pressure-overloaded LMS were associated with upregulation of inflammatory mediators and cytokines, many of which lied downstream of the NF-κβ transcription factor (Table 3.4) (Kuwahara et al., 2004). Inflammation is a major component of the remodelling response to pressure-overload (Vanderheyden et al., 2005). For example, IL-19 has been shown to attenuate aberrant cardiac remodelling following myocardial infraction in mice, and was significantly downregulated in pressure-overloaded LMS here (An et al., 2019).

Pressure-overload is known to induce changes in ECM composition including fibrosis (Crozatier & Ventura-Clapier, 2015). The expression of Col6a6, Col8a2, Eln, as well as Adarns2, and Tgfb3 genes were increased in the overloaded groups. However, there were no significant differences in the protein content of type 1 collagen compared to physiological load. As type 1 collagen deposition is part of the delayed wound response process (Xue & Jackson, 2015) it is possible that more time in culture is required for detectable differences. In support of this, collagen content of dog LMS cultured under isometric load using stretchers does not increase until day 7 of culture (Perbellini et al., 2018b).

3.4.6 Shared genes with same direction of expression

Cells have elaborate mechanisms to degrade or process misfolded proteins, which can accumulate following environmental stresses (Glembotski, 2008). Functional annotation showed that the endoplasmic reticulum protein processing pathway was commonly enriched in both pressure- and volume-overloaded LMS, and the expression of classical chaperons (Hsp90, Hspa5) as well as protein disulphide isomerases (Pdia3, Pdia4, Pdia6) was decreased in both compared to physiological load. Perturbation of the ability of the endoplasmic reticulum to process proteins, and particularly in the context of increased protein expression due to hypertrophy, can promote misfolded protein accumulation, endoplasmic reticulum stress, and contribute to progression into maladaptive cardiac dysfunction phenotypes (Wang et al., 2018).

Mechanical stimuli are converted to biochemical signals via mechanotransduction proteins (Kehat & Molkentin, 2010). Mechanical overload induced the expression of multiple mechanotransduction genes including integrins, focal adhesion kinases, and proteins located internally along the cardiac Z-line (Table 3.4). It is likely that the initial hypertrophic
response to the increased preload or afterload is mediated by these proteins (Kehat & Molkentin, 2010).

Brain natriuretic peptide (BNP) is a hormone synthesized and released by ventricular cardiomyocytes following mechanical stretch; BNP is the protein product of the Nppb gene. As the expression of BNP has a relatively good negative predictive value in HF, it was surprising to find Nppb significantly decreased in pressure- and volume-overloaded compared to physiologically loaded LMS. However, similar decreases in Nppb expression have been reported in cultured human LMS (Fischer et al., 2019) and studies have reported that BNP levels are highly variable depending on stage of disease. For example, in human subjects with essential hypertension BNP levels are initially decreased, which may suggest an impaired response of the natriuretic system to increased mechanical load (Belluardo et al., 2006). This framework, that BNP is not merely a marker but also a modulator of the remodelling response, is supported by studies which show that Nppb knockout in mice results in an exaggerated hypertrophic response (Kishimoto, Rossi & Garbers, 2001). Dysregulation of the natriuretic response could thus contribute to cardiac dysfunction (Gardner, 2003), particularly in the setting of chronic mechanical overload, as experienced here by LMS.

### 3.4.7 Shared genes with polarised direction of expression

Apart from Actn1, which codes for α-actinin, all shared genes between the overloaded groups had the same direction of expression. α-actinin is a spectrin superfamily with at least 4 isoforms expressed ubiquitously in skeletal and cardiac muscle as well as non-muscle cells. It is part of the sarcomeric apparatus and mediates myofibrillar assembly, organisation, and remodelling (Yu & Russell, 2005)(Manisastry, Zaal & Horowits, 2009). Interestingly, the expression of Actn1 is upregulated exclusively in hearts with depressed cardiac function (Hein et al., 2009). Actn1 expression was significantly upregulated in volume-overloaded but not pressure-overloaded LMS. Given its role in sarcomeric remodelling but also pattern of expression in disease, it is likely that Actn1 is a molecular marker:

- sensitive to nature of mechanical load, that is diastolic stress from high preload or systolic stress from high afterload and/or,
- of compensated (in pressure-overload) or decompensated (in volume-overload) cardiac performance

To further probe for this, we quantified the protein expression of α-actinin 1 and found that it was not significantly different between the three groups. However, it is possible that this is due to different rates of protein degradation. α-actinin 1 is broken down by calpain, a
Ca$^{2+}$-dependent protease (Hein et al., 2009), and the gene expression of calpain-3 (capn3) was significantly increased in volume-overloaded but not in pressure-overloaded LMS.

### 3.4.8 Limitations and future work

We aimed to develop a platform that can simulate pathophysiologically relevant mechanical load *in vitro* and use it to study the effects of load on myocardial phenotype. Although the method to apply mechanical load in LMS described here has greater physiological relevance than other methods (e.g. static stretch, auxotonic load, cyclic stretch), the 3EWK model is still a model – that is, a simplification of the circulatory load experienced by the heart. More advanced models should be examined including the 4-Element Windkessel, which in addition to Ra, Rc, and Ca, incorporates an inductor, L, to model the total arterial tree inertance (Stergiopulos, Westerhof & Westerhof, 1999). Furthermore, to simulate pressure-, or volume-overload here only afterload or preload were manipulated. *In vivo*, the distinction between such overloaded conditions may be less demarcated and more during the development of each disease, features of the other may be present in a dynamic and non-linear fashion (Opie et al., 2006).

We included basal levels of hormones to mimic the hormonal environment of the *in vivo* myocardium. However, these were always added in the same concentration, and were thus decaying across time (as they were supplemented once every 24 hr). *In vivo*, and particularly following alteration in CO (as would occur with altered afterload/preload) multiple feedback systems are in place to alter contractility, vascular resistance and maintain perfusion to peripheral organs (Pitoulis & de Tombe, 2019). Although the absence of these here means that greater degrees of causality can be drawn between load and phenotype (Pitoulis et al., 2019b), addition of more sophisticated feedback based neurohormonal systems comparable to those found *in vivo* could improve physiological relevance and disease modelling (see 6.1 for future projects).

All LMS were paced at 1 Hz during culture, which is considerably lower than the *in vivo* heart rate of ~ 300-500 bpm. As LMS are adult preparations, this was done similar to other culture platforms (Fischer et al., 2019)(Watson et al., 2019) to minimise oxygen and nutrient supply-demand disparities.

Perhaps the biggest limitation encountered in conducting the experiments described in this chapter was the low throughput of the system. Only a single Aurora was available and only one LMS could be cultured in the Aurora. Thus, for 3 day experiments, the max n number was 2 LMS per week. Moreover, the design of the Aurora was not intended for
chronic experiments, with multiple additional components needed to run it (stimulator, pump, incubator, custom chamber). In recognition of a) the limitations of running experiments within such a complicated set-up, but b) of the advantages of having a system that can recreate relevant mechanical load, we set out to develop, from scratch, a stand-alone device that could do what the Aurora did, better, more robustly, and cheaper; this is MyoLoop and is described in Chapter 4.
Chapter Four

MyoLoop: a bioreactor to streamline *in vitro* cardiac tissue electromechanical culture
4 MyoLoop: A bioreactor to streamline in vitro cardiac tissue electromechanical culture

4.1 Introduction

There are currently no commercially available experimental systems to culture cardiac tissue under the mechanical load experienced by the heart during the in vivo cardiac cycle. For all experiments described in Chapter 3 the Aurora apparatus had to be extensively modified. This is because the Aurora is not designed for chronic culture experiments but rather acute studies on freshly prepared tissue. As previously listed in Chapter 3, the following equipment was needed to run experiments:

- mN sensitive force sensor (part of the 300C dual mode muscle lever)
- nm sensitive length actuator (part of the 300C dual mode muscle lever)
- Incubator
- Computer and peripherals components
- Data acquisition systems (DAQ)
- Peristaltic pump
- Tissue pacer
- Software (custom LabVIEW programs)

Not only do all of these are needed, but they do not work in isolation. Instead, custom code, and system modifications are necessary for chronic experiments. For example, though the Aurora system provides tissue chambers, these did not work with LMS, and we had to create our own custom CNC-machined chamber (see 3.2.6). When all the components are put together, the price of a single unit can range between £35,000 and £70,000. In principle, more systems could be produced by purchasing multiple unitary components and replicating the entire set-up. Although such an approach could be beneficial in the short-term it is impractical, costly, and makes subsequent scale-up of the technology intangible.

A de novo system needed to be designed and developed to culture LMS in vitro. There were two main objectives in designing this:

a) Address problems encountered in previous culture set-ups
b) Minimise user complexity to enable easier intra- and inter-laboratory technology dissemination.

As the necessary skills (software development, computer aided designs (CADs), electronic prototyping, PCB design, CNC machining) to build the device were acquired in sync with the actual R&D, the entire process from prototype to first product took ~ 2 years.
The final device was named MyoLoop and will be referred to as such henceforth. A patent was filed by Imperial College London on 11th November 2019. The inventors were F.G. Pitoulis, P.P. de Tombe, and C.M. Terracciano.

4.2 Motivation and scope

The design of MyoLoop was driven by three specification categories. First, general culture specifications, which refer to settings that are essential for any chronic in vitro experiment. Second, culture parameters that have been shown to be advantageous for LMS culture, identified from experiments in previous culture set-ups, as described in (Watson et al., 2019). Third, general system specifications, which are MyoLoop settings that allow system reliability, ease-of-use, and improved user experience. These are described broadly in Table 4.1 and in detail throughout this chapter.

| Table 4.1: Specifications of an ideal bioreactor for chronic in vitro experiments |
|---------------------------------|----------------------------------------------------------------------------------|
| **Temperature & pH control:** physiological requirement to maintain temperature at 36 °C and pH at ~ 7.4 |
| **Sterility:** materials capable of withstanding disinfection protocols |
| **Biocompatibility:** materials should not be harmful to the LMS directly or indirectly via release of toxic by-products |
| **Oxygenation:** design must ensure adequate O₂ supply to the LMS |
| **Media recirculation:** continuous media agitation has been shown (Fischer et al., 2019) to be advantageous for LMS viability |
| **Mechanical load:** Muscle length control is necessary to recreate physiological relevant load (Chapter 3) |
| **Minimal LMS handling:** functional assays should be conducted in situ the culture set-up while the tissue is in steady state |
| **Self-sufficient:** minimise the need for external instruments |
| **Bench-top:** minimise clutter to permit easy access to LMS for mounting/unmounting and user-friendliness |
| **Lower cost:** to enable higher throughput |
| **Automation:** where possible automate experiments and analysis to minimise bias and improve repeatability of findings |
To meet these specifications, MyoLoop was designed from scratch. Where possible, components that would typically be obtained from third party companies were developed in-house to a) to fit directly within the desired device specifications, and b) to reduce cost.

**4.3 MyoLoop breakdown**

MyoLoop is divided into five main systems (Figure 4.1). These are in turn divided into subsystems, which in turn are divided into subsubsystems. These all interact and/or communicate with each other for the device to operate. The main systems are:

- Physical assembly
- Mechanical load controller
- DAQ
- Temperature regulation
- Stimulator controller

In the following sections the subsystems and subsubsystems technicalities are examined.

Where relevant, the rationale behind specific technical decisions and their link to biological experiments is provided.
Figure 4.1: MyoLoop system hierarchy

The MyoLoop bioreactor controls five systems which in turn control subsystems, and subsubsystems.

4.4 Physical assembly

This refers to the components that make up MyoLoop; that is the culture chamber, adaptors, linking parts (mounting rods,) as well as the mounting stand. We start by first examining these relatively to the standard culture set up used by our lab in the past.

4.4.1 The MyoLoop apparatus

The objective with MyoLoop was to create a self-contained device that could continuously recreate the cardiac cycle *in vitro* whilst meeting the necessary specifications for chronic *in vitro* cardiac culture (Table 4.1). Its components include:

- Chamber stand
  - Base plate
  - Heater plate
  - Force sensor mount
  - Chamber mount

- Culture chamber

- Connectors
  - Linear actuator and force sensor rods
  - Force sensor adaptor
  - Linear actuator adaptor

- Electronics enclosure box (see 4.8)

All physical assembly parts were sketched, rendered, and visualised in Fusion360 (Autodesk, FRA). The assembled MyoLoop is shown in Figure 4.2, while the physical assembly breakdown and 3D CAD model are shown in Figure 4.3. For manufacturing, each component was exported into .step format, and 2D diagrams drawn for each. These were used together to machine each component as summarised in Figure 4.4. For initial prototyping, parts were made by the Imperial College London Mechanical Instrumentation Workshop. Subsequently, components were machined by Facturee, DE. In the following paragraphs the rationale and technical features behind each component are briefly described.

The base plate is a rectangular shaped 3 mm thick 316 stainless steel platform onto which the linear actuator, culture chamber, and force sensor sit. The actuator is bolted in place by five M4 screws. On the opposite end is the force sensor mount. This consists of a slide fit key, a horizontal mount, and a vertical mount. The slide fit key is secured onto the base using two M3 screws. The horizontal mount, which has a negative of the slide fit key, is then positioned on top of it (Figure 4.3). This constrains the horizontal bracket in the lateral
direction \((z\text{-axis})\) but allows it to move in the horizontal direction \((x\text{-axis})\) (see Figure 4.3 for axis). During experiments, after adjusting for \(x\text{-axis}\) position, the horizontal bracket can be secured in place by tightening two M4 hex screws on either of its sides. The vertical mount is secured to the anterior end of the horizontal bracket and consists of a central oval shaped gap through which the force sensor comes through. This gap is large enough to allow for adjustment of the vertical position \((y\text{-axis})\) of the force sensor. In summary, the force sensor mount design enables coarse manual adjustments of its \(y\text{-}\) and \(x\text{-axis}\) position. This permits the system to accommodate different LMS lengths, which can vary between experiments and species.

Leg shaped rods were designed that connected to the force sensor and actuator adaptors on either side and extended to the inside of the chamber. These were used to suspend the LMS during culture, and were aligned on the \(z\text{-}\) and \(y\text{-axis}\), ensuring that the LMS contraction vectors were uniform along the \(x\text{-axis}\) alone. Both rods were made of 316 medical grade stainless steel to allow for autoclave sterilisation at the end of the experiments. The linear actuator can displace a load of up to 6 N. A 316 stainless steel 1.6 mm diameter rod, 51 mm in total length was designed, totalling \(\sim 0.77\) g, well within the actuator tolerance. The force sensor rod was made from 1 mm 316 stainless steel, 15 mm length, while the adaptor was made from nylon. This made the total weight of the rod-adaptor unit for the force sensor \(\sim 0.07\) g and minimised any changes in the force sensor mass characteristics.
Figure 4.2: MyoLoop physical assembly
A) Front view of assembled MyoLoop. B) Top view assembled MyoLoop.
Note: Cable connections with MyoLoop enclosure box (see 4.8) are not shown here.
Figure 4.3: MyoLoop physical assembly breakdown and CAD model
A) Each part was designed in Fusion360, machined, and then used to assemble MyoLoop. B) Exploded view of MyoLoop 3D model with labelled parts.
Design begins with parametric modelling of relevant features. Each part is then exported into .step format and 2D diagrams drawn for each in Fusion360. These are used in conjunction to CNC-machine each part. Parts are finally assembled using nuts and bolts.

Figure 4.4: Flowchart from CAD to physical assembly

Design begins with parametric modelling of relevant features. Each part is then exported into .step format and 2D diagrams drawn for each in Fusion360. These are used in conjunction to CNC-machine each part. Parts are finally assembled using nuts and bolts.
The culture chamber is the unit that harbours the LMS during the experiment. It is made up of three parts: the chamber lid, the chamber, and the chamber floor. These are made from polycarbonate, PEEK, and 316 stainless steel respectively. The height of the chamber is 30 mm, while outer and inner dimensions are 75 × 50 mm and 50 × 25 mm in length × width respectively. Thru holes are found 20 mm high along its anterior and posterior sides, allowing the rods from the actuator and force sensor to enter the chamber. There are four \( \frac{1}{4}-28' \) UNF threads for luer-type connectors, and two 4.1 mm thru hole for carbon electrodes on either lateral side of the chamber (Figure 4.2B). Culture media was recirculated through the luer-type connectors. As the thru holes are inflow/outflow their height determines the amount of fluid that fills the chamber and based on the chosen dimensions, the total chamber volume was 25 ml. One design flaw was that the open actuator and force sensor thru holes present a possible path for the entry of microorganisms, which could contaminate the culture set-up during experiments. To mitigate this, the chamber wall thickness was designed to be 12.5 mm \(((\text{outer chamber length} - \text{inner chamber length})/2 = (75-50)/2 \text{ mm})\), thus forming a solid gap between the outside environment and the media inside the culture chamber.

Another design consideration was to make the culture chamber detachable to allow sterilisation at the end of the experiments. Thus, the chamber was not permanently mounted on the base. Instead, a heater plate was screwed on the underside of the base, and the chamber bolted on the heater plate using four M2 screws (Figure 4.3). Thus, the culture chamber could be isolated from the rest of the system for sterilisation at the end of the experiment and bolted on the heater plate at the start of the experiment.

Although the 25 ml of media inside the chamber could maintain the LMS viable for at least 1 day without exchange, a media bottle, which stores media was always connected to the chamber and recirculated media via a peristaltic pump, tubing, and the luer-type connectors. The same principle was used for the Aurora experiments (see 3.2.8) However, we found that occasionally the excess media in the bottle (> 25 ml of chamber volume) would fill the chamber past the thru holes, leading to overflow and leakage. As the same peristaltic pump was used for media inflow and outflow with the same flow rates, the problem was attributed to capillary tension. In the final version of the culture chamber, we incorporated a capillary break to prevent water from ‘climbing’ out of the chamber through the actuator and force sensor thru holes (Figure 4.5).

To maintain the contents of the culture chamber at physiological temperature the chamber floor was made from 316 stainless steel (Figure 4.3). When the chamber was mounted on the heater plate at the beginning of culture, the chamber floor was in contact with
the heater plate. On the underside of the heater plate, a silicone heating pad was adhered and controlled by the MyoLoop electronics. This heated the heater plate and consequently the chamber base by means of convection, ultimately keeping the culture media to the desired temperature. This design discarded the need for an incubator, the use of which was impractical due to a) the presence of multiple sensitive electrical components (e.g. force sensor, actuator) which must be maintained in non-humid conditions, and b) for easier accessibility to the tissue. The control software and electronics of the temperature control are discussed in 4.6.1).
Figure 4.5: Capillary break to prevent media leakage
A) MyoLoop 3D model. When using an external media bottle, the chamber would occasionally overfill due to capillary tension. A capillary break was designed below the level of the thru holes to mitigate this. B) Cross-section of culture chamber showing leak sources and capillary break.
4.5 Mechanical load controller

The core operation of MyoLoop was the recreation of the in vivo work loop on in vitro cardiac tissue, in this case LMS. As discussed in Chapters 1 & 3, in vivo the heart generates pressure concurrent with simultaneous changes in volume. The corresponding vectors in a pseudo-3D LMS are force and length. Like in the Aurora set-up (Chapter 3) a 3EWK mathematical model was used to simulate the cardiac cycle in MyoLoop.

For mechanical load to be applied to the LMS in the form of precision work loops, two components are required: a) a force sensor, and b) a linear actuator. The force sensor detects the force that the LMS is generating. The length-actuator shortens or stretches the LMS in sync with force generation via application of the predictions made by the 3EWK model. Importantly, these predictions depend on the force generated by the LMS and the input model parameters (Ra, Re, Ca).

For such a set-up to operate, a DAQ module comprising analogue-to-digital converter (ADC), amplifiers and filters, as well as an actuator controller to program the motions of the actuator are needed. Additionally, software and algorithms to integrate and synchronise these, run the 3EWK model, enable user-input and real-time visualisation of acquired data are also required.

In MyoLoop, an F30 or F50 Harvard Apparatus (Cambridge, UK) force sensor, a V-273.440 Voice-Coil Actuator (Physik Instrumente, DE) with the C-413.2GA Motion Controller (Physik Instrumente, DE) actuator controller, and a USB-6001 DAQ device (National Instruments, USA) were used. The software for these was programmed in LabVIEW 2019 (National Instruments, USA). The remainder of this section will focus on the LabVIEW code and algorithms.

4.5.1 Programming a cardiac work loop

LabVIEW is a graphical programming language used for automation, data acquisition and measurement. A LabVIEW program or subroutine is called a virtual instrument (VI). Each VI consists of two parts: a front panel, and a block diagram. The front panel is the graphical user interface (GUI). The block diagram contains the source code. A VI can be used in the source code of another VI where it is termed a subVI.

In MyoLoop, a LabVIEW program is used to run the 3EWK, control the force- and length-data acquisition, the actuator position and length waveforms, as well as all user commands relevant to these functions, such as file I/O (i.e. saving data), data display, entering 3WK input parameters. These processes are controlled by multiple subVIs and
subsubVIs all embedded within a main VI called *main.vi*. Only some of these subVIs will be discussed in detail here. Upon completion of the LabVIEW code, a distributable format of the software was deployed using the National Instruments application module. This resulted in an executable program which could be installed via a standard USB device containing the MyoLoop installer, and the MyoLoop executable application. This was favourable as it allowed MyoLoop to be run without installing any other National Instrument applications, as would be normally required to run LabVIEW.

The *main.vi* did not control the following:

- temperature,
- stimulator frequency and pulse width

These were controlled by a RaspberryPI microcontroller and a Python program; they are discussed later in this chapter.

### 4.5.1.1 Program design

The *main.vi* program was designed using a queued message handler design architecture (QMH) (Figure 4.6). This is a software architecture allowing multiple sections of code to run simultaneously by having multiple *while loops* each responsible for a task executed at the same time. Three different types of while loops are found in this design:

- **Master loop**
- **Message handler loop**
- **Slave loops**

There is a single *master loop*, a single *message handler loop*, and multiple *slave loops*. The *master loop* generates messages in response to user-input. These are processed by the *message handler loop* and send messages to the *slave loop(s)*, which are instructed to perform the user-requested task. For example, in response to the button ‘Start data acquisition’ on the GUI front panel being pressed by the user (Figure 4.7 and Figure 4.11), the *master loop* generates a message named ‘Data acquisition button pressed’, which is sent to the *message handler loop*. The *message handler loop* processes the message and in turn sends the message ‘DAQ ON’ to the *slave loop* responsible for the data acquisition task.

The *master loop* produces but does not receive messages. *Slave loops* can also generate messages, which are then processed by the *message handler loop* and distributed to the same or different *slave loop(s)*. This enables synchronisation of tasks carried out by different *slave loops*. For example, once *slave loop A* has performed task X, message ‘X finished’ is sent by *slave loop A* to the *message handler loop*, which then sends message ‘start Y’ to *slave loop B*,
which then starts task Y. Furthermore, messages can be sent to multiple different slave loops by the message handler loop after the former has received a single message. To illustrate this, from the data acquisition example before, together with the message ‘DAQ ON’ being sent to the data acquisition slave loop, the message ‘Start timer’ could also be sent to a timer slave loop, which starts a pomodoro timer that saved data iteratively after a particular unit of time set by the user has elapsed (e.g. every 300 s). Complex message networks can be established in this way which allow for parallel task running and process automation. The MyoLoop source code and a video walkthrough of it is shown in Figure 4.7.

The QMHL architecture was chosen for several reasons. Firstly, multiple sections of code can execute simultaneously. This is vital for our application as it permits the MyoLoop user-interface to remain responsive to the user even if other sections of code are being run in the background. For instance, if DAQ is started, the user can record force generated by the LMS while simultaneously taking control of the actuator to manually stretch or relax the tissue, as would be desired in a Frank-Starling experiment. Secondly, processes could be easily automated and streamlined by passing specific messages after certain tasks have completed execution. Thirdly, messages can carry any data type (not just string), such as array of double-point floating (DBL), single precision floating (SGL), etc. This allows data to be dynamically passed to and from loops for real-time processing, saving, and subsequent analysis.
**Figure 4.6: Queued message handler design**

This is a template of the QMH design. A sample of messages in three colours are drawn to and from loops for illustration. Notice that the *message handler loop* receives messages (arrows in black, green, red) from the *master* and *slave loops* and directs messages to *slave loops* (arrows in green, and red). *Slave loops* can also pass data between them (arrow in blue). The *master loop* only sends data based on user input commands (e.g. display data, start acquisition, etc.).
Figure 4.7: MyoLoop source code
A) MyoLoop QMH design. Notice multiple loops and network of arrows directing the flow of messages and data. B) QR code of LabVIEW back-end walkthrough.
4.5.1.2 Data acquisition slave loop

Data acquisition was carried out in the DAQ slave loop. The USB-6001 DAQ card was configured to acquire a finite number of samples in two analog channels at 1 kHz sampling frequency. These corresponded to the analog output of the force sensor amplifier and actuator respectively. They were set-up in low-to-high transition trigger mode and were wired to the TTL pulse generated by the MyoLoop stimulator (see 4.6.2). The total duration of acquisition was given by:

\[
\text{duration}_{\text{acq}} = \frac{1}{f_s} \times n_s
\]  

(15)

Where \(f_s\) and \(n_s\) are the sampling frequency and number of samples respectively. Total duration of acquisition had to be less than the stimulation interval for the acquisition to complete before the arrival of the next TTL trigger. To accommodate the potential for users to change the stimulation frequency while maintaining complete data acquisition, \(n_s\) was limited to 80% of stimulation interval, as shown in Table 4.2.

<table>
<thead>
<tr>
<th>Stimulation Interval / (s)</th>
<th>Stimulation Frequency / (Hz)</th>
<th>Sampling Frequency (f_s) / (Hz)</th>
<th>Sampling Duration / (ms)</th>
<th>Number of samples (n_s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>0.5</td>
<td>1000</td>
<td>1600</td>
<td>1600</td>
</tr>
<tr>
<td>1000</td>
<td>1</td>
<td>1000</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>500</td>
<td>2</td>
<td>1000</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>333</td>
<td>3</td>
<td>1000</td>
<td>267</td>
<td>267</td>
</tr>
<tr>
<td>250</td>
<td>4</td>
<td>1000</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>200</td>
<td>5</td>
<td>1000</td>
<td>160</td>
<td>160</td>
</tr>
</tbody>
</table>

The data acquired in these channels by the DAQ slave loop was sent to the 3EWK slave loop for processing and running of the 3EWK model. In following sections, 800 data points of the standard 1 Hz stimulation frequency are used for illustration purposes.

4.5.1.3 3EWK slave loop

The 3EWK slave loop was automatically started when data acquisition was started and was responsible for producing a length waveform based on the force generated by an LMS in its previous beat and the 3EWK parameters set by the user in the LabVIEW GUI (Figure
It received data from the DAQ slave loop and send data to the Actuator slave loop as well as to data display and file I/O loops (Figure 4.8).

The 3EWK model was run its original pressure-volume form, by converting the force-transients to stress ($\sigma$) and then pressure ($P$) using Laplace’s law according to equation (3) as previously described in 3.2.7. Nominal values for healthy heart wall thickness ($h$) and radius ($r$) were used and these were changed automatically for different species by the software when the user selected the desired species in the LabVIEW GUI (Figure 4.11). The pressure waveform was fed into the 3EWK model which calculated the instantaneous rate of change of volume, $dV/dt$ or in the electrical analogy, the instantaneous rate of change of current $dQ/dt$. This was subtracted from the previous volume and was iterated for the entirety of the pressure waveform to yield a volume waveform. The volume waveform corresponded to the LV volume transient for any given pressure. A conditional statement differentiated between the systolic and diastolic phase, such that:

$$\text{If } LVP > AOP:$$

$$\frac{dV}{dt} = f(P, Rc, Ra, Ca)$$

$$\text{If } LVP < AOP:$$

$$\frac{dV}{dt} = 0$$

where $AOP$ is aortic pressure and $LVP$ is left ventricular pressure, $dV/dt$ is instantaneous rate of change of voltage, $P$ is pressure, and $Rc, Ra, Ca$ are the 3EWK model parameters.

In other words, the conditional statement determined whether the aortic valves are open or closed; this means that that the 3EWK does not predict a diastolic refilling phase as $dV/dt = 0$ for diastole. Thus, when the diastolic conditional statement is fulfilled, all predicted volumes correspond to the same volume, equal to end-systolic volume (ESV). To stretch the LMS back to diastolic length, and thus end-diastolic volume (EDV), a simple linear diastolic refilling phase was incorporated into the model, where the speed of refilling back to EDV was parametrized. The source code of this diastolic model is the final magnified panel of Figure 4.7.

The predicted LV volume transient was converted to muscle length using a sphere as geometrical model of the ventricle according to:

$$V = \frac{4}{3} \times \pi \times r^3$$

$$r = \sqrt[3]{\frac{3}{4} \times \frac{V}{\pi}}$$

155
\[ L_o = L_r \times \% \text{ strain} \]  \hspace{1cm} (18)

\[ L = \frac{r}{r_o} \times L_o \]  \hspace{1cm} (19)

Where \( V \) is volume, \( r \) is radius, \( r_o \) is end-diastolic radius, \( L \) is LMS length, \( L_o \) is end-diastolic tissue length, and \( L_r \) is LMS resting length. Thus, given a predicted volume timecourse, \( V \), a predicted length timecourse, \( L \), would be obtained and subsequently actuated on the LMS (equations (16) to (19)). A visual description of a single run is shown in Figure 4.8.
Figure 4.8: 3EWK algorithm implementation
Signal processing on the left and underlying VI on the right, represented by their respective custom icon. Notice two different examples of diastolic refilling (slow, and fast) for the same systolic profile on lowest panel, set by changing the diastolic speed parameter of the linear diastolic refilling model.
4.5.1.4 Actuator loop and actuator timer

The actuator slave loop carried out two functions:

- real-time control of the actuator position, and
- configuring the actuator for TTL-triggered 3EWK waveform motions

The former allowed the LMS to be stretched or relaxed to the desired muscle length at any point during the experiment with nm precision. Biophysical assays such as Frank-Starling experiments or positioning the LMS to the desired muscle length were therefore possible via simple commands on the user interface panel (see Figure 4.11). This eliminated the need for manual adjustment of LMS length (e.g. using callipers as was previously done in the Aurora).

The muscle length time course predicted by the 3EWK was sent to the actuator slave loop. It was then loaded in the volatile memory of the C-413 actuator controller. The Physik Instrumente LabVIEW waveform library was used to perform the motion profiles. Specifically, the length transient was loaded in waveform format in the wave table of the wave generator of C-413. The wave table can store up to 4096 waveform data points at any given time. Waveforms are called using wave commands, which are then output based on the actuator hardware configuration. In MyoLoop, the actuator was configured to output the waveform when the controller’s input trigger digital line was high. Thus, when the TTL pulse was sent from the stimulator module, the actuator would begin its motion. Importantly, this was different to the DAQ hardware configuration (set to begin acquisition after a low-to-high TTL transition) as the actuator waveform points were only being outputted when the digital line was high. As such, for the actuator movement to be synchronised to the stimulation pulse and interval and DAQ trigger, the MyoLoop stimulator needed to generate an ON pulse (TTL = HIGH) equal to the expected duration of the waveform motion (Figure 4.9). The default servo-cycle of the V-273 actuator was 202.66 μs and the number of servo cycles to output a single waveform point can be modified to an integer multiple of this. A servo-cycle multiplier of 5 was chosen, leading to an output rate of 1.0133 ms per sample. This slight peculiarity meant that although the analog input channels were sampled at 1 kHz the actuator output rate was 987 Hz, and the ON pulse duration was set by:

$$\text{duration}_{\text{pulse}} = \frac{1}{f_s} \times n_s$$  \hspace{1cm} (20)

Where $f_s = 987$ Hz, and $n_s$ is dependent upon the stimulation rate as shown in Table 4.2. Setting the correct pulse ON duration was vital for synchronisation of the output waveform to
the electrical stimulus and the next waveform. For example, if the pulse ON duration was 800 ms (corresponded to 1 kHz for $n_s = 800$) a recurrent rightward drift of 9.86 samples or 10.64 ms per waveform would occur. The pulse ON was generated by the stimulator unit described in 4.6.2.

The waveform generator module of the C-413 controller was designed by PI for applications with highly repetitive motions; it was therefore highly suitable for chronic \textit{in vitro} experiments where at 1 Hz stimulation frequency 60 waveforms are generated per minute continuously for at least 72 hours. Furthermore, it enabled the output of precisely timed motion profiles, without the need of real-time operating system which would require further components (e.g. National Instruments Real-Time Module) and raise the price.

\textbf{4.5.1.5 Feedback based muscle length control}

As previously detailed, LMS are continuously remodelling during culture (Chapter 3). Like in the Aurora, a closed-feedback loop was designed to ensure that the waveform being performed by the actuator matched the predictions of the 3EWK. This is demonstrated in Figure 4.10, which shows an LMS performing the green work loop, but the 3EWK model predicting the red work loop. As the LMS remodels and its force generation changes, for the same 3EWK parameters a different length waveform is predicted, causing the offset between the green (actual) and red (predicted) waveforms in Figure 4.10.

To ensure the actuated waveform was based on the latest contraction data (and thus converging with the 3EWK predictions), two ways to load the predicted length waveform data to the wave table of the C-413 controller were programmed. Firstly, by manual user input – that is, the user would simply click the ‘Load Waveform Data’ in the user-interface (Figure 4.11). Secondly, using an automatic timer that loads the data after a user-defined period has elapsed. In both modes the length waveform data from the latest beat were loaded. The sequence of steps of this process is shown in Figure 4.10.

To load the length waveform to the wave table, an iterative algorithm requiring ~5 s for an 800-point waveform was used. This meant that the lowest waveform update time resolution was ~5 s. This was a step forward from the Aurora length-actuator set-up (Chapter 3) where the clamping of the length was done via analog control and required ~30 s.

However, the ~ 5 s still meant that beat-to-beat resolution was not captured in the length plane. Furthermore, during this 5 s period, whilst the waveform was being loaded to the C-413 volatile memory, the LMS was beating under isometric contraction (Figure 4.10).

Although more advanced methods for controlling the actuator, such as using field
programmable gate array (FPGA) were contemplated the relatively low time resolution and general stability of LMS in culture discouraged their pursuit.

The 3EWK model used in MyoLoop did not account for the internal resistance of the ventricle (De Tombe & Little, 1994). Tissue shortening clips force-generation according to force-velocity curves (Sonneblick, 1962), with maximum force production occurring at isometric load. This created a problem because when a length-waveform was first imposed on a LMS, force-generation was immediately altered due to tissue shortening, causing the 3EWK predictions to change as well.

To illustrate this point, consider a LMS beating under isometric load and the 3EWK predicting a length waveform (not yet actuated) based on the isometric force data. As soon as, the length waveform is actuated on the LMS, force generation decreases due to tissue shortening. This causes the 3EWK predictions to change based on the new ‘clipped’ force. A constant offset is created in this manner as each length-waveform is based on the previous beat. A simple iterative algorithm was coded to correct this, thus accounting for the internal resistance of the ventricle. Any time length waveform data $y$ from a force transient $x$ was loaded to the controller, the algorithm would carry out two iterations. In the first iteration, lasting a single beat, the length waveform $y$ was actuated and the next force transient $x+1$ was acquired. The $x+1$ force transient was then fed to the 3EWK, which output a second length waveform $y+1$. This was then actuated on the tissue. The $y+1$ waveform was actuated on the LMS until waveform generation stopped manually by the user, a new waveform was loaded manually by the user, or the elapsed time of the automatic timer reached the user-defined target time, automatically loading a new waveform.
Figure 4.9 the 3EWK model is predicting: Control of actuator output

Actuator output is either ON or OFF based on the state of the TTL digital line. Output begins once TTL pulse is ON (=1) and ends when TTL pulse pulls low (=0). For a length waveform consisting of 800 data points, the length waveform output lasts for ~810 ms, corresponded with an output rate = 986 Hz. Notice bipolar stimulator pulse to trigger tissue contraction synchronised with actuation of the length waveform.
Figure 4.10: MyoLoop updating length waveform on LMS
A) GUI of MyoLoop before, during, and after a length waveform update. Initially, the green work loop is being actuated on the LMS, but red work loop is being predicted by the 3EWK. After pressing the ‘Load waveform’ button or the target time elapsing, the new (red) waveform is actuated on the LMS. Whilst the waveform is updating the LMS beats isometrically for ~ 5 s (middle panel). B) QR code of this process during an experiment.
Figure 4.11: LabVIEW graphical user interface
LabVIEW MyoLoop GUI. From here, the user can control the amount of stretch to the tissue, the mechanical load mode (isometric or Windkessel), DAQ commands, file I/O commands, as well as timers for saving data and automatically updating the waveform once a defined period has elapsed. The different coloured traces correspond to predicted and actual length waveforms in red and green respectively. Note: simulated data are shown in this Figure.
4.6 Electrical assembly

The MyoLoop electrical assembly included the temperature controller, the stimulator, and the force sensor amplifier. Electrical circuits of these were designed in the web-based circuit designer EasyEDA. Initially, breadboard prototypes were developed for rapid testing and troubleshooting (Figure 4.14A). Subsequently, the final working prototype was converted to Gerber files and used for the manufacturing of MyoLoop printed circuit boards (PCBs); these were ordered from JLCPCB, CHN (Figure 4.15). All electrical circuits were designed such that two MyoLoop chambers could be run from a single PCB.

In the following sections, the different modules of the breadboard/PCB and some of the essential technical aspects of each are discussed. The final circuit schematic is shown in Figure 4.13 and will be used as a reference.

To control the settings of the custom electrical components (e.g. temperature, stimulation pulse width, stimulation frequency) a second GUI was programmed in Python using the tkinter library. This GUI is shown in Figure 4.12, and its code is available in Appendix 1. In sum, MyoLoop had two GUIs: a) LabVIEW GUI, which controlled the force and length data acquisition and generation, file I/O, saving, displaying, and processing data (Figure 4.11), and b) Python GUI, which controlled the peripheral components (Figure 4.12).

For the initial validation experiments described in 4.9, the breadboard prototype of MyoLoop was used as shown in Figure 4.14. Subsequently, all the electronics were enclosed within a custom aluminium box as outlined in 4.8.
Figure 4.12: Python general user interface
This is the MyoLoop Python GUI, used to control the temperature of the culture chamber, as well as the stimulation frequency and pulse width.
Figure 4.13: Electrical circuit schematics

MyoLoop electrical system is split into temperature controller and heaters, power for the RaspberryPI microcontroller, amplifiers and filters for the force sensor, stimulator and TTL triggers for DAQ and actuator control. Each of these is encircled in its box above.
**Figure 4.14: Electrical circuit breadboard and initial set-up used for validation experiments**

A) MyoLoop breadboard. The breadboard has the circuits of Figure 4.13 and was subsequently used to develop the PCB (Figure 4.15) which was enclosed within an enclosure box (Figure 4.20). B) Initial set-up used for validation of MyoLoop. Notice that the set-up consists of the physical assembly, the C-413 actuator controller, the NI USB-6001 for data acquisition, as well as the breadboard and microcontroller. The computer monitor running the LabVIEW and Python GUI is not shown.
Figure 4.15: Final PCB
A) PCB designed on EasyEDA. B) PCB with some of the electrical components soldered on.
4.6.1 Temperature regulation

The temperature of the media in the culture chamber was kept within physiological range by the temperature controller unit. This comprised a temperature sensor, a heat delivery circuit, the control algorithms written in Python and relevant buttons in the Python GUI, as well as the stainless steel heating plate and chamber floor as previously described (4.4.1).

4.6.1.1 Temperature sensor

A rapid response time 10 kΩ negative temperature coefficient thermistor with polyester insulation (TE Connectivity, USA) was selected as the temperature sensor. A voltage divider circuit consisting of a fixed 10 kΩ resistor \( R_{\text{fixed}} \) in series with the thermistor was assembled (Figure 4.13). The fixed resistor was chosen to be equal to the nominal resistance of the thermistor (10 kΩ) as this provided the highest sensitivity for the interested temperature range (Figure 4.16). The tolerance of the thermistor allowed temperature accuracy to ± 0.5 °C. The voltage across the thermistor, \( V_{\text{out}} \), changed as a function of the thermistor’s resistance \( R_{\text{thermistor}} \) according to:

\[
R_{\text{thermistor}} = \frac{V_{\text{out}} \times R_1}{V_{\text{in}} - V_{\text{out}}} \tag{21}
\]

Where \( V_{\text{in}} = 3.3 \, V \), \( R_1 = 10 \, k\Omega \). \( V_{\text{out}} \) was sampled at 1 Hz using a 10-bit analog-to-digital converter (MCP3008, Microchip Technologies, USA) controlled by the RaspberryPI via serial peripheral interface (SPI) communication protocol according to (Friedrich, 2020). The calculated value for \( R_{\text{thermistor}} \) was then used to compute the temperature using the Steinhart-Hart equation:

\[
\frac{1}{T} = \frac{1}{T_0} + \frac{1}{\beta} \ln \left( \frac{R_{\text{thermistor}}}{R_0} \right) \tag{22}
\]

Where \( T \) is the temperature, \( T_0 \) is room temperature in kelvin (298.15 K), \( \beta \) is the thermistor’s temperature coefficient (3976), and \( R_0 \) is the resistance of the thermistor at room temperature (10 kΩ). During culture the thermistor was immersed in the culture media inside the culture chamber through one of the luer-type connectors.

4.6.1.2 Heat delivery

To heat the chamber, a 7.5 W silicone heating pad was adhered to the underside of the stainless-steel heater plate. As the chamber floor was also made of stainless steel, heat was conducted between them. When an external media bottle was also used, a 15 W silicone
heating pad was wrapped around the media bottle. Both heating pads were controlled using a simple proportional negative feedback algorithm according to:

\[ Heater_{drive}(t) = \theta \times error(t) \]  

(23)

Where \( Heater_{drive}(t) \) is related to the voltage applied to the heating pads at any given time, \( t \), as a function of the error at time \( t \), \( error(t) \), between the desired temperature, \( T_{desired} \), and the current temperature at time \( t \), \( T_{current}(t) \), and the Boolean variable \( \theta \), where:

2538 If \( T_{desired} - T_{current}(t) > 1 \):
2539 \( error(t) = 1 \)
2540 \( \theta = 1 \)
2541 Else if \( 0 < T_{desired} - T_{current} < 1 \):
2542 \( error(t) = T_{desired} - T_{current} \)
2543 \( \theta = 1 \)
2544 Else:
2545 \( \theta = 0 \)

Pulse width modulation (PWM) was used to control the voltage applied to the heating pad. 1000 Hz pulses were generated by the RaspberryPI and applied to the base of an NPN-transistor (ON Semiconductors, USA) ((Figure 4.16). The duty cycle of the PWM was equal to the \( Heater_{drive}(t) \), and thus 100% when \( error(t) > 1 \), 0% when \( error(t) < 0 \), and between 0 - 100% when \( 0 < error(t) < 1 \). For example, if \( error(t) = 1.5 \, ^\circ C \), \( Heater_{drive}(t) = 1 \) corresponded with 100% duty cycle and 12 V (max) applied to the heating pad and if \( error(t) = 0.5 \, ^\circ C \), then PWM duty cycle = 50% and 6 V were applied to the heating pad. This algorithm ensured rapid response of the system when heating up and stability when the desired temperature was reached. 220 and 411 Ω base resistors were used to control the current to the base of the NPN transistors, ensuring full saturation during the ON phase of each pulse of the PWM.

The desired temperature was set in the MyoLoop RaspberryPI GUI as shown in (Figure 4.12). The error and current temperature were also displayed and updated at 1 Hz frequency. The python code can be found in Appendix 2.
Figure 4.16: Simulations of selected thermistors response to temperature

A) Change in $R_{\text{thermistor}}$ as a function of temperature. This is an exponential decay, with decay constant, $\alpha = 4.39\% / ^\circ C$, according to thermistor datasheet.

B) Simulations of output voltage ($V_{\text{out}}$) of the voltage-divider for different values of the fixed resistor ($R_{\text{fixed}}$), with $V_{\text{in}} = 1 V$. Different lines correspond to different values of the fixed resistor ($R_{\text{fixed}}$) in the voltage divider circuit. In MyoLoop $R_{\text{fixed}}$ was set equal to $1R_{\text{thermistor}}$ (green line), as this provided the highest sensitivity, $dV/dT$, over the interested temperature range 30 - 40 °C.
**4.6.2 Stimulator controller**

The stimulator controller unit delivered current pulses that paced the LMS and set the TTL triggers for actuator movement and DAQ. Carbon electrodes entered from the lateral sides of the culture chamber wall (Figure 4.2) suspended by M4 screws, met the media filling the chamber and field stimulated the LMS.

**4.6.2.1 Pulse properties**

The electrical stimulus delivered to the LMS was designed to meet several specifications deemed optimal for chronic *in vitro* cardiac tissue culture.

The shape of the stimulus was chosen to be biphasic with an inter-pulse delay (Figure 4.17). Each stimulus consisted of a first positive and a second negative pulse of equal duration separated by a zero-current phase. This shape was chosen as it minimises non-reversible Faradaic reactions at the electrode-media interface, reducing harmful by-products and electrode degradation (Tandon *et al.*, 2009a). The total duration of the stimulus was adjustable via the Python GUI from 6 – 22 ms (Figure 4.12), and the inter-pulse delay was purposefully made non-adjustable and always equal to 2 ms. The amplitude of the pulses was controlled by a potentiometer dial on the MyoLoop enclosure box (see below and Figure 4.20). In Figure 4.16 pulses with different amplitude and width characteristics are shown.

A range of frequencies (0.5-, 1-, 2-, 3-, 4-, and 5 Hz but not frequencies in between) were programmed into the stimulator controller unit and were selectable via the Python GUI (Figure 4.12). To ensure data acquisition does not stop, a change in stimulator frequency requires a corresponding change in the DAQ sampling interval (see 4.5.1.2); thus, when the stimulator frequency was changed in the Python GUI the user also needed to change the sampling frequency in the LabVIEW GUI; this had to be done manually by the user and not automatically by the software.

The biological rationale behind the stimulation pulses is to mimic the QRS complex, which initiate ventricular contraction. Accordingly, the stimulus delivered to the LMS was synchronised to the TTL pulse ON delivered to the actuator by the RaspberryPI (Figure 4.9 and section 4.5.1.4), ensuring that LMS shortening via the actuator occurred in sync to contraction.

**4.6.2.2 Constant current power op-amp**

The stimulator was built around the LT1970 chip (Analog Devices, USA) (Figure 4.13 and Figure 4.15). The LT1970 is a power op-amp with externally controlled current limits,
set separately for the sourcing, and sinking voltage. It can output \( \pm 500 \text{ mA} \) and operates with 2 MHz response between setting the current source or sink limits and the amplifier output.

The source and sink current limits are set by controlling the voltage input at the \( V_{\text{SRC}} \) and \( V_{\text{SNK}} \) pins of the op-amp respectively, with the relationship between input voltage and output current being linear. In MyoLoop, \( V_{\text{SRC}} \) and \( V_{\text{SNK}} \) were always set to 3.3 V (voltage output of the RaspberryPI pins). To control the amount of current delivered by the stimulator (i.e. amplitude of the pulse), an adjustable linear voltage regulator circuit using the LM317T was used. This controlled the power supply to the LT1970 and by extension the current output of the op-amp as shown in Figure 4.19. The output of the linear regulator, and thus current delivered to the LMS, was controlled using the potentiometer knob (Figure 4.20), and was equal to:

\[
V_{\text{out}} = V_{\text{ref}} \times \left(1 + \frac{R_2}{R_1}\right) + I_{\text{adj}} \times R_2
\]

\[24\]

\[
V_{\text{out}} = 1.25 \times \left(1 + \frac{R_2}{150 \ \Omega}\right) + 0.1 \times R_2
\]

\[25\]

Where \( V_{\text{ref}} \) is the internal reference of the linear regulator and equal to 1.25 V, and \( I_{\text{adj}} \) is the current flowing through the constant resistor \( R_1 \) and equal to 0.1 A. Then, \( V_{\text{out}} \) depends on the value of \( R_2 \) alone, which corresponds to position of the wiper in the potentiometer ranging from 0 to 1000 \( \Omega \) for a 1K potentiometer. In this manner \( V_{\text{out}} \) ranged from \( \sim 2 \) to 10 V.

To ensure synchronisation of LMS stimulation and actuator movement, the RaspberryPI output two digital waves simultaneously. The first wave, consisted of the actuator pulse ON TTL, which activated the actuator to perform the length waveform loaded on the C-413 controller (Figure 4.9). The duration of this pulse dependent on the stimulator frequency as previously described. At the same time, the \( V_{\text{SRC}} \) pulse, interpulse delay, and \( V_{\text{SNK}} \) pulse were generated by the RaspberryPI. These controlled the LT1970 and triggered the output of the pulse that depolarised the LMS. The actuator and stimulator waves needed to be timed relative to one another, but also to the next iteration of themselves. For example, if the stimulator pulse and actuator pulse lost sync then there would be electromechanical decoupling of the LMS. Likewise, if the waves were not synchronised with their next iterations, then there would be a progressive offset in the actuator motion. The two waves and their relationship are shown in Figure 4.9 and Figure 4.18.

A general problem with using software-based approaches to control measurement and automation systems, is that by default, many operating systems, including Debian on which
the RaspberryPI runs, are not deterministic – that is, they do not operate in real-time. The result is that in running programs, the amount of time required for the same program to execute the same function in subsequent iterations may be different (longer or shorter) compared to previous iterations. This is known as jitter, and it complicates precise and reliable timing. To address this, we utilised the pigpio package, a Python module that communicates with the general-purpose input/output pins (GPIO) RaspberryPI’s daemon. Using pigpio, the RaspberryPI GPIOs can generate digital waves that are accurate and precise up to 1 μs. The pigpio was used to generate the digital waves to both op-amp and actuator controller, satisfying the synchronisation requirements. This can be appreciated by Figure 4.18, which shows analysis of the stimulator and actuator pulses in an oscilloscope. The python code running the stimulator code can be found in Appendix 3.
Figure 4.17 Bipolar electrical signal from stimulator unit on oscilloscope
A) 10 ms electrical signal. B) 22 ms electrical signal. Amplitude of pulse is adjusted by the potentiometer dial of the LM317 adjustable linear regulator. Pulse width is controlled by Python GUI. For all figures the oscilloscope was set to 10 ms, 2 V per division with $R_{load} = 1.2 \, k\Omega$. 
Figure 4.18 Stimulator and actuator waves and GUI
A) Synchronisation between stimulator pulse (bipolar) and actuator ON pulse. Oscilloscope set to 10 ms per division. B) Zoomed in scope of (A) at 2.5 μs per division. A 10 μs delay was intentionally programmed between stimulator and actuator pulse. This was corrected in the LabVIEW algorithms by shifting the length waveform by 0.1 ms to the right when loading it to the actuator controller. C) Zoomed out scope of (A) –set to 250 ms per division. Notice synchronisation between multiple subsequent pulses.
Figure 4.19 Adjustable linear voltage regulator to control current output of LT1970

Adjustable linear regulator circuit used to control power to LT1970 and by extension its current output. As R1 = 150 Ω and R2 anything between 0 and 1000 Ω, Vout could range from ~ 2 to 10 V according to equation (25). Smoothing capacitors and diodes are not shown here.
4.7 Force sensor amplifier

LMS force generation was measured by the F30 and FT50 (Harvard Apparatus, UK) force transducers. The F30 was used in the initial validation set-up. It was later discontinued by Harvard Apparatus and the FT50 was used to replace it.

The F30 and FT50 are full-bridge Wheatstone strain gauges. A strain gauge is an electrical circuit which contains resistive elements, the resistance of which varies with application of strain. In a full-bridge Wheatstone, there are 4 resistors, arranged as a parallel pair of two resistor in series, typically drawn in a diamond configuration. When a voltage source is applied to the circuit, the voltage difference ($V_{out}$) between the parallel branches can be measured as a function of applied strain. Thus, mechanical strain is converted to a measurable electrical signal, $V_{out}$. To measure $V_{out}$ an amplifier based on the INA125 instrumentation amplifier (Texas Instruments, USA) was designed as shown in Figure 4.13.

The gain of the amplifier as set by the following equation according to the technical datasheet:

$$Gain = 4 + \frac{60 \, k\Omega}{R_g}$$  \hspace{1cm} (26)

With $R_g$ being the resistor controlling the amplification gain. To calculate the required gain, the F30 or FT50 specifications were used together with the sensitivity range of the USB-6001 DAQ system (Table 4.3).

**Table 4.3: F30 and FT50 specifications and necessary amplification**

<table>
<thead>
<tr>
<th>Specification</th>
<th>F30</th>
<th>FT50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity at full scale</td>
<td>10</td>
<td>29</td>
</tr>
<tr>
<td>(mV/V)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excitation voltage (V)</td>
<td>5 V DC</td>
<td></td>
</tr>
<tr>
<td>Output voltage per g of force (mV/g/V)</td>
<td>0.33</td>
<td>0.58</td>
</tr>
<tr>
<td>$V_{out}$ range (mV)</td>
<td>0-50</td>
<td>0-145</td>
</tr>
<tr>
<td>USB 6001 DAQ</td>
<td>± 10 V</td>
<td></td>
</tr>
<tr>
<td>Required gain (G)</td>
<td>200</td>
<td>690</td>
</tr>
</tbody>
</table>

For 200-fold and 690-fold gain the gain resistor ($R_g$) was set to 306- and 87 Ω respectively according to the technical specifications of the amplifier and equation (26).
4.8 MyoLoop instrument box

The MyoLoop PCB, NI USB-6008, and RaspberryPI were all placed within an aluminium instrument case. The front and back panel dial positions and drill sizes were designed on Fusion360, and CNC machined on the panels provided with the box. All dials were purchased from RS Components, UK.

The instrument box allowed us to have a compact version of MyoLoop with switches and knobs, shielding any exposed wires of the initial prototype. This increased device accessibility and user-friendliness. The MyoLoop box is shown in Figure 4.20 and the final fully assembled device in Figure 4.21.
Figure 4.20 MyoLoop instrument box
A) MyoLoop box as seen from the front. Note: labels were deliberately stuck non-straight. B, C) Inside of MyoLoop box seen from the back and front respectively. Notice MyoLoop PCB, RaspberryPI, and NI USB-6001 DAQ are all placed inside the box.
Figure 4.21 Final MyoLoop set-up
A) This is the final MyoLoop product. Notice LabVIEW and python GUI on monitor on the right, MyoLoop box and physical assembly centre front, C-413 left of the monitor screen, media bottle and peristaltic pump on the left.
Following MyoLoop development we set the following objectives:

a) Demonstrate the ability to parametrise preload and afterload

b) Assess the robustness and stability of the device.

c) Demonstrate a proof-of-concept application of LMS using MyoLoop

For objective (a) we conducted a series of short acute experiments in which LMS were stretched to different diastolic length (preload) and looped with different values of Ra, Rc, and Ca.

For objective (b) we conducted 72 hr culture experiments on adult rat LMS, with the same mechanical load parameters as that of Chapter 3. We considered a successful culture if we were able to maintain the LMS alive for 3-days with similar functional performance profile (i.e., contractility) as that obtained in the Aurora.

For objective (c) we performed cardiotoxicity testing over 3-day culture, using sunitinib. Sunitinib is a tyrosine kinase receptor inhibitor used in the treatment of multiple solid tumours. Despite its therapeutic potential, it has been shown to cause LV dysfunction in ~ 20 % of patients (Truitt et al., 2018), while in vitro studies on both hiPSC-CMs EHTs and pig LMS have shown decreased tissue force generation and viability within 24 hr and 48 hr culture respectively (Truitt et al., 2018) (Miller et al., 2020). We cultured adult rat LMS for 3 days with or without 1 μM sunitinib, the physiological relevant concentration of the drug during anticancer therapy (Truitt et al., 2018). We set out to determine the effects of chronic cardiotoxic agent exposure on LMS contractile performance.

4.9.1 Methods

4.9.1.1 LMS preparation

LMS were prepared from adult SD rats, trimmed, and had rings attached perpendicular to the main myocardial fibre direction as described in 2.3 and 2.4.

4.9.1.2 Culture media

Culture media was prepared fresh at the start of each experiment; media was replaced on each culture day as described in 2.3.4.

4.9.1.3 3D printed rings

3D printed rings were printed as described in 2.5. The ring design shown in Figure 3.9D was used for the experiments conducted here.
4.9.1.4 Acute force-length loops

Prepared LMS were stretched to three different % stretches (110%, 116%, and 122%) whilst inside the MyoLoop. At each diastolic length, the LMS would be allowed to loop using two different 3EWK parameters. These were 1) \( R_c = 0.5, R_a = 5, C_a = 0.5 \), and 2) \( R_c = 0.3, R_a = 3, C_a = 0.9 \).

4.9.1.5 Culture of LMS in MyoLoop

Prepared LMS were transferred to the MyoLoop set-up immersed in slicing Tyrode’s solution inside a 150 mm x 15 mm Falcon petri dish (Corning, UK). The MyoLoop stimulator was turned off, and the MyoLoop LabVIEW program initiated. When the MyoLoop program first runs, the actuator position automatically references its position via an impulse move lasting ~ 1 s. After referencing, the actuator position was set such that the distance of the actuator rod to the force sensor rod was ~ 7 mm, allowing the LMS to be mounted without overstretching.

LMS were picked up from their rings using forceps and mounted between the rods. The stimulator was turned on, data acquisition started, and the LMS progressively stretched until passive force, visualised in the LabVIEW GUI, started to increase – that is, until no longer slack. The LMS was then stretched to 2.1 \( \mu \)m SL and allowed to beat for 15 min under isometric load. All beats were saved during these 15 min. This can be appreciated in Figure 4.24, which shows physiological looping of LMS starting after ~ 700,000 acquired data points. As 800 data points were acquired per beat (see 4.5.1.2), this equates to ~ 15 min of continuous monitoring (700,000/ (800 × 60)).

After 15 min the 3EWK algorithm was initiated and the LMS started looping based on the same 3EWK parameters and preload as those used in Chapter 3. These corresponded to physiological preload (2.1) \( \mu \)m and afterload (\( R_a = 7, R_c = 0.5, C_a = 0.5 \)). The diastolic filling phase was set to 0.6 via the LabVIEW GUI corresponding to a 200 ms linear ramp protocol after isometric relaxation, which brought the LMS back to end-diastolic length (same as 3.2.7). Identical diastolic phase is critical in the study of remodelling as slow LV filling and prolonged isovolumic relaxation have been linked to development of HF with preserved EF (Burchfield, Xie & Hill, 2013).

To ensure that the LMS shortening matched force production, the V-273 timer (Figure 4.11) was set to 600 s. Thus, the waveform actuated on the LMS was updated every 10 min, The data acquisition logging timer was set to 300 s; 1 beat every 5 min was saved to file I/O. All beats were visualised via the LabVIEW GUI.
Media was recirculated using an external MasterFlex peristaltic pump (see Figure 4.14 and Figure 4.21) and 3/8 in platinum-curated peristaltic tubing (Cole-Parmer, UK) with inflow/outflow rates equal to 50 ml/min and connected to a 500 ml external media bottle using a double perfusion system gassed with 95% O₂ 5% CO₂. The temperature of the chamber was kept at 36 °C, and the LMS stimulated at 1 Hz and 6-12 ms pulse width. Both were controlled using the Python GUI.

4.9.1.6 Sunitinib cardiotoxicity culture

10 mM stock of sunitinib malate (Sigma-Aldrich, UK) was prepared in dimethyl sulfoxide (DMSO). This was then diluted in the culture media. The final concentration was 1 μM and sunitinib was added on each media exchange (day 0, 1, and 2). As the final dilution was 1:10000, DMSO-related toxicity was minimal (Truitt et al., 2018).

4.9.1.7 Data analysis

Force and length data were continuously monitored on MyoLoop and saved in .tdms format by default. A simple LabVIEW .tdms reader was coded, which allowed visualisation of data (Figure 4.24), as well as conversion of .tdms files to .txt or .csv for further analysis. The tdms files were first converted to .csv and the data analysed as described in 2.10.1.2.

To determine the relationship between force production and LMS stroke length, Δlength, during culture, a linear regression was fit to the active force – Δlength data (Figure 4.23).

4.9.2 Statistics

Statistical analysis was performed in Prism8 as described in 2.13. P<0.05 was considered statistically significant.

4.9.3 Results

4.9.4 Acute work loops ins MyoLoop

To illustrate the ability of MyoLoop to parametrise both preload (via diastolic stretching) and afterload (via the 3EWK) we performed a series of work loops at different preload and with different afterload. These experiments were conducted in the completed MyoLoop set-up (Figure 4.21) and the results shown in Figure 4.22.

4.9.5 MyoLoop for chronic in vitro LMS culture

The MyoLoop DAQ enables continuous monitoring of the force generated by the LMS and the length waveform actuated to it at any given point during culture (Figure 4.23). LMS active force progressively increased during culture from day 0 to day 3, whereas passive
force remained relatively constant (Figure 4.23A). The force trendline was consistent and similar to that of the Aurora set-up (Figure 3.13), including the U-shaped force response at the beginning of culture (Figure 3.13 & Figure 4.23A), validating objective (b). However, the absolute force generated by LMS in MyoLoop was lower. This was a constant force offset, observable from day 0 of culture, and was attributed to the different force sensors used in each set-up (F30 or FT50 by Harvard Apparatus in MyoLoop vs. 300C dual-mode muscle lever by Aurora Scientific).

As aforementioned, MyoLoop was operating based on a closed feedback loop. The rationale behind this has been detailed in 3.4.1 and 4.5.1.5, and the results of this can be appreciated in Figure 4.23B,C and Figure 4.24. As LMS force production changed during culture, the ΔL also changed in sync to reflect that. Algorithmically, this was accomplished by updating the length waveform every 600 s based on the force transient at the 600 s timepoint. This is depicted in Figure 4.24, where all the work loops performed by the LMS during the 72 hr culture (logged to file memory every 5 min) are shown. Interestingly, and as observed by others (Fischer et al., 2019) LMS contractility declined following media exchange; this is shown with arrows in Figure 4.24.

To further assess MyoLoop’s robustness as well as the reproducibility of my findings, MyoLoop was used in a series of 72 hr culture experiments by Dr Danika Hayman. These experiments were conducted after a 1-day training course, which included device walkthrough and acute experiments. The major problem encountered by Dr Hayman during her experiments was the leakage of media fluid outside the culture chamber. This problem, described in 4.4.1, was addressed in the subsequent culture chamber by incorporating the capillary break (Figure 4.5). Dr Hayman was able to reproduce the results presented here, further validating both MyoLoop and the LMS as a novel cardiac preparation for chronic in vitro culture.

**4.9.6 Drug testing with MyoLoop**

LMS were cultured for 72 hrs with 1 μM sunitinib. In contrast to others (Truitt et al., 2018) (Miller et al., 2020), we did not find significant differences in LMS contractility, stroke length, or time to peak during culture compared to LMS cultured in control media (no sunitinib) (Figure 4.25). It is possible that the superior and more physiological relevant environment to which the LMS is exposed during culture in MyoLoop better preserves myocardial phenotype. Thus, more prolonged experiments may be required to observe differences, particularly considering that both (Truitt et al., 2018) and (Miller et al., 2020))
observed these at an earlier timepoint (24 h and 48 h respectively). Use of different species could also contribute to result disparity; adult rat LMS were used whereas (Truitt et al., 2018) used hiPSC-CMs EHTs and ((Miller et al., 2020)) pig LMS.
Figure 4.22 Acute work loops
LMS were loaded onto the MyoLoop chamber and stretched to three different % stretches from LMS resting length (110%, 116%, and 122%). At each diastolic length two work loops were performed with 3EWK parameters of 1) \( R_c = 0.5, R_a = 5, C_a = 0.5 \), and 2) \( R_c = 0.3, R_a = 3, C_a = 0.9 \).
Figure 4.23 Functional data of LMS cultured in MyoLoop
A) LMS active and passive force production during 72 hr culture. B) Stroke length (Δlength) during 72 hr culture. C) Active force-ΔL linear regression. Note: One force transient was acquired every 5 min, but only one data point for each day of culture is shown here. N = 4. Data shown as mean ± SEM. D) Force-length loops during 72 hr culture in MyoLoop for an LMS equal to 10.0 mm in length stretched to 2.1 μm preload.
Figure 4.24 72 hr force-length data using MyoLoop TDMS reader

A) Force-length waveform graph. A decrease in LMS force production causes the stroke length to decrease, maintaining afterload constant. Media exchanges causes LMS force production to decrease. B) Overlay of all work loops obtained during 72 hr culture.
Figure 4.25 Effect of sunitinib on LMS contractile performance

A) Active and passive force of LMS cultured in control or with sunitinib for 72 hr. B) ΔL of LMS cultured in control or with sunitinib for 72 hr. C) Time to peak of LMS cultured in control or with sunitinib for 72 hr. N = 4. Data shown as mean ± SEM.
4.10 Discussion

We have described the development of a novel bench-top device for chronic *in vitro* culture of adult cardiac tissue. Platforms to culture LMS *in vitro* have been produced by us (Watson *et al.*, 2019) and other laboratories (Ou *et al.*, 2019)(Fischer *et al.*, 2019)(Qiao *et al.*, 2018). The two central features unique to MyoLoop are a) the recreation of the *in vivo* pressure-volume electromechanical events *in vitro* as force-length loops and b) being self-contained. Other technologies have cultured LMS unloaded (Ou *et al.*, 2019), under isometric (Watson *et al.*, 2019)(Qiao *et al.*, 2018), or auxotonic load (Fischer *et al.*, 2019).

Additionally, they often require multiple external components to set-up, run the culture, but also the subsequent functional (e.g. contractility) experiments; the only external component required by MyoLoop was a peristaltic pump.

4.10.1.1 Streamlining physiologically relevant LMS culture

Recreation of pressure-volume relationships *in vitro* is possible with commercially available laboratory equipment (De Tombe & Little, 1994). However, as we have shown in Chapter 3 and discussed here these require extensive coding and customisation. Working knowledge of software design but also manufacturing techniques (e.g. 3D CAD modelling for culture chamber etc.) and assembly is therefore necessary. As this increases the complexity to set-up and run experiments, it limits the potential for technique dissemination. Although manufacturing of MyoLoop is challenging, once assembled and the relevant software installed, the device is ‘plug-and-play’ and does not need further user input or customisation.

4.10.2 Real-time manipulation of mechanical load

The application of physiologically relevant mechanical load in MyoLoop means that both preload and afterload are parametrised, but also that these can be altered in real-time during culture. In past set-ups from our lab (Watson *et al.*, 2019) preload can only be set at the beginning of culture, whereas afterload is non-modifiable and equal to isometric load.

Similarly, in the auxotonic spring-like set-up of (Fischer *et al.*, 2019) although preload can be changed during culture, changing the afterload requires changing of the spring against which the LMS is contracting; this cannot be done during culture and is time-consuming.

The ability to change preload and afterload enables many applications. Firstly, different mechanical loads can be applied to the LMS during *in vitro* culture. A simple case concerns the initial application of high afterload to simulate hypertension over 48 hr, followed by the application of low afterload to unload the LMS and determine whether progression to pathological mechanical phenotypes (as shown in Chapter 3) can be averted by mechanisms...
of reverse remodelling (Ibrahim et al., 2010)(Pitoulis & Terracciano, 2020). Secondly, direct adjustment of mechanical load enables both acute and terminal stretch-based functional experiments to be conducted in situ the bioreactor. As previously described, this preserves the LMS at a steady-state and increases reproducibility of data.

4.10.2.1 Continuous monitoring of LMS contractility

All force transients generated by the LMS are captured by the MyoLoop DAQ and all or fewer (dependent on the Data Logging Timer) can be saved to the file I/O for future analysis. This was a major improvement as it allows continuous monitoring of LMS behaviour and viability.

4.10.2.2 Bench-top operation

Except for a peristaltic pump, which recirculated the culture media, MyoLoop did not require any further external apparatus to function. Data acquisition and processing, temperature control, and stimulation where all incorporated into the designs. Development of these technologies from scratch, without relying on third-party companies, meant that a) the cost to produce one device was significantly lowered and b) that each device was autonomous. In contrast, other systems including that developed by us (Watson et al., 2019) and others (Ou et al., 2019) required incubators, stimulators, peristaltic pump and DAQ systems for subsequent contractility measurements, while that of (Fischer et al., 2019) and (Qiao et al., 2018) required an incubator as well as DAQ systems for contractility measurements at end-point culture.

4.10.3 Limitations

Although MyoLoop was designed as a dual system, capable of running two culture chambers with a single PCB and C-413 controller, this was still lower throughput than the standard culture chamber of our lab (Watson et al., 2019) or the auxotonic culture system of (Fischer et al., 2019), which could accommodate 4 and 8 LMS respectively. Another limitation was the lack of communication between the Python GUI and the LabVIEW GUI. For example, starting/stoping acquisition of force data did not start/stop when electrical stimulation was started/stoped from the Python GUI. Instead, if electrical stimulation was changed in the Python GUI, the user then had to manually change it in the LabVIEW GUI as well. Similarly, changing stimulation frequencies required the user to first stop DAQ in the LabVIEW GUI, then change stimulation frequency in the Python GUI, and then change DAQ frequency and start DAQ in the LabVIEW GUI.
Although the constant current stimulator was designed to have two equal but opposite pulses (see 4.6.2), it was observed that following 72 hr of culture the positive electrode (i.e. electrode connected to the LT1970 output) was undergoing redox reactions suggesting a current leakage. This meant that following six-to seven 72 hr experiments, electrodes needed to be replaced as they failed to stimulate the LMS. The addition of the LM317T adjustable linear voltage regulator (4.6.2.2) ameliorated this to an extent, given that current could be set to lower values increasing electrode lifespan. However, more work is needed for the sink and source charges to be balanced.

MyoLoop was faster in actuating the length waveform predicted by the 3EWK on an LMS than the Aurora set-up. However, the waveform was controlled digitally – that is, 3EWK predictions were uploaded to the wavetable of the C-413 and then actuated. ~ 5 s were needed to load the waveform, during which the LMS was beating isometrically, and this process was repeated every time the waveform was updated (typically 600 s as described in 4.9.1.1). Although the effects of ~ 5 s isometric beats during 72 hr culture (total of ~ 35 min isometric beating) on LMS remodelling are likely to be minimal this loading algorithm means that like in the Aurora, beat-to-beat variations in length waveform are not captured by the system. Others have addressed this by using FPGA-arrays (Garrett et al., 2019), which are considerably faster in analog-digital operations. As LMS beat-to-beat variability is minimal, we did not pursue this approach.
Chapter Five

Investigation of transmural cardiac mechanical properties using intact LMS
5 Investigation of transmural cardiac mechanical properties using intact LMS

5.1 Introduction

Most experiments described in this thesis concerned chronic in vitro culture of LMS. However, the intermediate complexity of the model makes them an ideal preparation for acute physiological experiments. In this chapter, we leverage the unique method of LMS preparation, which involves sequential cutting from subendocardium to subepicardium, to investigate whether mechanical heterogeneity exists across the LV wall.

The ventricular myocardium is a tissue with regional and transmural structural and functional heterogeneity. Differences in electrical properties including activation and distribution of ion channels, Ca²⁺ dynamics, and action potential propagation have been described in multiple studies already (Cordeiro et al., 2004)(Liu & Antzelevitch, 1995)(Clark et al., 1993), yet transmurality of cardiac mechanics has remained relatively unexplored. Still, understanding mechanical properties across the LV wall is critical. Transmural heterogeneity in mechanical properties can impact cardiac work and ejection, and their loss may contribute to pathological progression in HF (Haynes et al., 2014). Additionally, transmural data may be relevant for clinical decision making and prognosis as mid-wall fractional shortening has been shown to be a better predictor of cardiovascular outcomes than ejection fraction or shortening of other LV free wall regions (Wachtell et al., 2010).

Although research on the cellular and molecular basis of such differences has begun, data are conflicting. The LV subendocardium has been shown to develop both higher active tension and passive tension than the subepicardium in some studies (Cazorla et al., 2005)(Cazorla, Le Guennec & White, 2000) but not in others (Haynes et al., 2014)(van der Velden et al., 2011)(Stelzer et al., 2008). Importantly, most studies to date have been conducted on permeabilised preparations or isolated cardiomyocytes. The former provide insight into the subcellular mechanisms of transmural variability but the physiological responses of the whole cell are lacking. Likewise, intact isolated cardiomyocytes are devoid of the effect of other cardiac and non-cardiac cell populations, the ECM, and the whole tissue response in regulating contraction (Pitoulis et al., 2019b).

We set out to investigate whether intrinsic mechanical differences exist in LMS prepared from different layers (subendocardium, midmyocardium, subepicardium) of the adult rat LV wall. The organotypic nature of the preparation meant that transmural mechanical properties
could be investigated within the setting of an intact cardiac tissue with electrical, metabolic, contractile, and structural properties of the native *in situ* heart. Our objectives were:

a) Determine whether transmural differences in mechanics are present and intrinsic to each cardiac layer, and

b) Uncover the structural and molecular basis of any such differences.

### 5.2 Methods

#### 5.2.1 LMS preparation

LMS were prepared from adult 11–15-week-old SD rats (350-470 g) using vibratome as described in 2.3 and 2.4.

#### 5.2.2 LMS from different layers of the LV wall

Due to the nature of slicing, LMS were prepared in sequence starting from subendocardium, then midmyocardium, and finally subepicardium. The thickness of the rat LV wall allowed on average 6 LMS to be prepared per heart. LMS 1 & 2 were pooled together as subendocardium, 3 & 4 as midmyocardium, and 5 & 6 as subepicardium. Henceforth, these will be referred to as endocardium, midmyocardium, and epicardium respectively.

#### 5.2.3 Laser diffraction experiments

The SL-% stretch relationship of LMS from different layers of the LV wall was determined by laser diffraction experiments as described in 2.8.1 and 2.10.5.

#### 5.2.4 Force-Ca$^{2+}$ dose-response

To assess the sensitivity of LMS from different LV wall layers to extracellular Ca$^{2+}$ ([Ca$^{2+}$]o), force-Ca$^{2+}$ dose-response experiments were performed. Half-log increasing Ca$^{2+}$ concentration solutions were prepared by diluting 1 M BioUltra Ca$^{2+}$ (Sigma-Aldrich, UK) in normal Tyrode’s. 1.8 mM Ca$^{2+}$ was also included in the dose-response, as this is the standard amount of Ca$^{2+}$ present in the normal Tyrode’s solution (Table 5.1).

These were the first functional experiments to be performed and aimed at identifying the sensitivity, defined as the as the Ca$^{2+}$ at which half max force is generated (EC$_{50}$), of endocardial, midmyocardial, and epicardial LMS to Ca$^{2+}$. The EC$_{50}$ specific to each layer could then be used during the Frank-Starling experiments (see below), to enable assessment of the LMS response to stretch while controlling for any transmural differences in the response to Ca$^{2+}$.
After preparation, LMS were transferred to the organ bath and mounted between the strain gauge and micromanipulator as described in 2.7. Initially, the organ bath contained oxygenated 1.8 mM [Ca\(^{2+}\)]_o Tyrode’s solution and was kept at 25 °C. After mounting the LMS electrical stimulation was started at 1 Hz, 20-30 V amplitude and 10-20 ms pulse width, and the temperature of the solution raised to 36 ± 0.5°C. The micromanipulator was used to stretch the LMS to a SL of 2.1 μm using muscle length as a surrogate and measured by the video camera, as described in 2.7.1. Diastolic length was then left unchanged throughout the experiment.

The LMS was allowed to beat in 1.8 mM [Ca\(^{2+}\)]_o for 6 min or until steady state was reached, after which superfusion was switched to a randomly chosen Tyrode’s solution containing an unused [Ca\(^{2+}\)]_o. This process was repeated for all [Ca\(^{2+}\)]_o. At the end of the dose-response, LMS were tested again at 1.8 mM [Ca\(^{2+}\)]_o Tyrode’s solution. If the active force was <60% of the active force generated by the LMS during the first run with the 1.8 mM [Ca\(^{2+}\)]_o solution, then the LMS was excluded from further analysis. Data were analysed manually (see 2.10.1.1) for determination of twitch force amplitude at each [Ca\(^{2+}\)]_o and a sigmoidal log(agonist) vs. response (variable slope with four parameters) model was fit to the data as described in 2.10.3.

From these experiments the following parameters were obtained:
- EC\(_{50}\): [Ca\(^{2+}\)]_o at which half-max force is produced
- Hill slope: \(H\) coefficient of equation (9)
- Maximum force: max force generated by LMS at any [Ca\(^{2+}\)]_o
- Force span: the difference between max and min force generated by LMS at any [Ca\(^{2+}\)]_o

**Table 5.1: Half-log Ca\(^{2+}\) solutions**

<table>
<thead>
<tr>
<th>Dose</th>
<th>Ca(^{2+}) Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
</tr>
<tr>
<td>1</td>
<td>10(^{-3.5})</td>
</tr>
<tr>
<td>2</td>
<td>10(^{-3.0})</td>
</tr>
<tr>
<td>3</td>
<td>10(^{-2.7})</td>
</tr>
<tr>
<td>4</td>
<td>10(^{-2.5})</td>
</tr>
<tr>
<td>5</td>
<td>10(^{-2.0})</td>
</tr>
</tbody>
</table>
5.2.5 Frank-Starling experiments and linear regressions

LMS were transferred to the organ bath and mounted between the strain gauge as described in 2.7. This was filled with oxygenated Tyrode’s solution containing $10^{-2.54}$ M Ca$^{2+}$ (correspondent to the Ca$^{2+}$ EC$_{50}$ of LMS, described in the results section). Field electrical stimulation was delivered to the LMS at 1 Hz, 20-30 V biphasic excitation voltage, 10-20 ms pulse width via platinum electrodes on opposite ends of the organ bath, and the temperature of the organ bath slowly raised to $36 \pm 0.5 ^\circ$C.

After ~ 10 min in the organ bath the isometric Frank-Starling response was investigated by progressively stretching the LMS to muscle lengths correspondent to SL of 2.00-2.10-, 2.20-, 2.25-, 2.35- and 2.40 µm. Once the desired muscle length was reached the force generated by the LMS was measured for 5 min or at until steady state.

To quantify the sensitivity of LMS from each layer of the ventricular wall to stretch, a linear regression was fit to the active force – SL data and the slope of the linear regression lines compared. The method by which this was done has been described in 2.10.2.

5.2.6 Immunohistochemistry

LMS were stained with cardiac troponin T, caveolin 3, vimentin, and Hoechst 33342 as described in 2.8.2, and Table 2.4, and visualised under 20X confocal microscopy as described in 2.8.3.

Cardiomyocyte area, length, width, and density were analysed blinded by Waseem Hasan, BSc, as described previously in 2.10.4.

Cardiomyocyte dispersion, a measure of variability of structures from a main axis of direction, was automatically quantified using the caveolin 3 staining x20 magnification confocal images and ImageJ’s directionality analysis tool. Within an LMS image, there is a main cardiomyocyte direction, with cardiomyocytes normally distributed around this main axis. High dispersion means that the number of cardiomyocytes that deviate from the main axis is increased and corresponds to the standard deviation of a gaussian distribution fitted by the directionality tool (Figure 5.1)

5.2.7 Picrosirius red staining

Picrosirius staining was used to quantify the collagen content of LMS from different cardiac layers. This was done in collaboration with Dr Samuel Y. Boateng and Nicolas G. Clavere at University of Reading, School of Biological Sciences, UK.

Six adult SD rat were sacrificed, their hearts explanted, blocked in Optimal Cutting Temperature (OCT) media, and stored at -80 °C for 24 h. Then, using a cryostat at -23 °C, the
OCT blocks were sectioned in 7 μm transverse sections, and these deposited on slides, which were stored at -80 °C until further processing.

Prior to staining, sections were first fixed in Bouin solution for 15 min inside the fume hood, and then washed with tap water for 15 min, both at room temperature. The Picrosirius red staining kit (ab150681, Abcam, UK) was used to stain the sections for 1 h at room temperature inside the fume hood, by following the manufacturer’s instructions. This allowed visualisation of collagen content. Stained slides were differentiated in acidified water, twice, with two dips. They were then washed and dehydrated three times in 100% Ethanol before being cleared in Xylene (Sigma-Aldrich, UK) for 5 min.

A brightfield microscope (Nikon TE200, UK) was used for visualisation of the stained sections. 10 pictures per area of interest per section were acquired, and two sections analysed for every animal.

To quantify the % area covered by picrosirius red the brightfield original images were first converted to RGB format. A threshold previously calibrated to cover most of the staining was then applied to the picture, and the area covered by the stain measured using ImageJ’s analysis tools.

5.2.8 ProQ diamond and Sypro Ruby stain

Sarcomeric protein and phosphoprotein content was characterised on LMS using ProQ Diamond and Sypro Ruby stain. These experiments were performed in collaboration with Dr Mary Papadaki and Prof Jonathan A. Kirk from the Department of Cell and Molecular Physiology at Loyola University, USA.

LMS from the endocardium, midmyocardium, and epicardium were quickly snap frozen in liquid N2 and stored at -80 °C until further processing. Myofilament fractions were prepared from each LMS, and run on a SDS-PAGE 4-12 % gradient gel (20 μg per lane) at 180 V for 45 min as described in (Papadaki et al., 2018). The gel was placed in 100 ml fixing solution (50% methanol, 10% acetic acid) and stained with ProQ Diamond (Invitrogen, USA) for 75 min by following the manufacturer’s instructions. Finally, it was de-stained with a 20% acetonitrile, 50 mM sodium acetate, at pH of 4.0.

The gel was then first visualised at 580 nm using a Typhoon scanner (GE Healthcare, USA). This allowed quantification of phosphorylated sarcomeric proteins. For total protein content, the gel was stained again overnight with Sypro Ruby (Invitrogen, USA), de-stained the next day with 10% methanol, 7% acetic acid, and visualised again using the Typhoon
scanner at 619 nm wavelength. Images were analysed in ImageJ, and the transmural gradient of phosphorylated to total sarcomeric protein ratio calculated.

5.2.9 Statistics

All statistical analysis was performed in Prism 8. For comparison of regression lines obtained from endocardial, midmyocardial, and epicardial LMS in the laser diffraction and active force–SL data, analysis of covariance (ANCOVA) test was used as described in (GraphPad Prism, 2017). For analysis of statistical differences in contractility (Frank-starling, 
Ca$^{2+}$, EC$_{50}$, Hill slope, force span), data obtained from confocal images, and sarcomeric protein content and phosphorylation, one-way analysis of variance (ANOVA) was used with Tukey’s post-hoc. P <0.05 was considered statistically significant. The following symbols were used for the group comparisons:

- *, **, ***, ****: p<0.05, p<0.01, p<0.001, and p<0.0001 midmyocardium vs. endocardium
- ^, ^^, ^^^, ^^^^^: p<0.05, p<0.01, p<0.001, and p<0.0001 epicardium load vs. endocardium.
- #, ##, ###, ####: p<0.05, p<0.01, p<0.001, and p<0.0001 midmyocardium vs. epicardium.
Figure 5.1 Analysis of cardiomyocyte dispersion
A) x20 magnification caveolin 3 stained confocal image used for analysis of cardiomyocyte dispersion. The image is split into 4 quadrants by the ‘Directionality’ tool and the main axis of direction and standard deviation determined (64.3° ± 8.75 here). B) Directionality histogram and results of image in (A).
5.3 Results

5.3.1 Transmural SL - % stretch relationships

The ventricular wall has been shown to operate at different SLs during the cardiac cycle in vivo (Cazorla, Le Guennec & White, 2000)(Rodriguez et al., 1992). As we controlled muscle length during our experiments, we first set out to identify whether LMS from different layers of the LV wall have different SL-% stretch relationships. The data specific to each layer were then used for functional and structural experiments. This was critical to ensure that LMS from different layers of the ventricular wall were stretched to the same SL.

If the SL - % stretch relationship was different, then the same % stretch would result in a different SL in each layer, compromising our independent variable. No significant differences were found in the SL-% stretch slopes between endocardial, midmyocardial, and epicardial LMS (Figure 5.2). The R² values were 0.60, 0.64, and 0.66 for endocardium, midmyocardium, and epicardium respectively.

5.3.2 Force-Ca²⁺ dose response for assessment of transmural LMS Ca²⁺ sensitivity

Cardiac muscle force generation is a function of extracellular Ca²⁺, [Ca²⁺]o. Thus, if the response of each layer to [Ca²⁺]o was different, for example as a result of different ion channel densities (Li et al., 2002), assessing mechanical properties using a solution with the same [Ca²⁺]o could mask intrinsic differences in contractility. Prior to the Frank-Starling experiments we performed isometric force-Ca²⁺ dose response experiments with LMS stretched at a preload of 2.1 μm SL.

Ca²⁺ sensitivity, the [Ca²⁺]o at which half-maximum force is generated (EC₅₀), obtained from the sigmoidal force-Ca²⁺ dose response curve was not significantly different between LMS (Figure 5.3). Furthermore, the Hill slope, which describes the steepness of the response, the maximum developed force, and the force span were not significantly different either (Figure 5.3). Practically, these experiments yielded a known Ca²⁺ concentration, equal to the experimentally determined EC₅₀ (10⁻².⁵⁴ M), at which all Frank-Starling experiments could reliably be conducted, and the mechanical properties of each layer investigated while controlling for Ca²⁺.

These results suggest that the response of LMS from different layers of the LV wall to Ca²⁺ is uniform. In permeabilised non-failing human cardiac preparations, and in contrast to our findings, the endocardium is more sensitive to Ca²⁺ than the epicardium (i.e. active force-Ca²⁺ relationship is shifted leftward)(Haynes et al., 2014). However, in such experiments the
preparation is not intact, and the response of sarcomeric components to Ca$^{2+}$ alone is examined.
Figure 5.2: Transmural SL-% stretch relationships and residual plot
A) The SL-% stretch relationship was examined using laser diffraction experiments on freshly prepared LMS from the LV endocardium, midmyocardium, and epicardium. B) Residual plots of the linear regression lines. N = 6. Figure adapted from (Pitoulis et al., 2020) under CCL.
Figure 5.3: Force-Ca$^{2+}$ dose response curves
A) Active force-[Ca$^{2+}$]o. B, C, D, E) Log EC$_{50}$ of [Ca$^{2+}$]o, Hill slope, max force, and force span of endo-, mid-, and epicardial LMS. N = 8, 7, 6 respectively. Data shown as mean ± SEM and median ± IQR in E. Figure adapted from (Pitoulis et al., 2020) under CCL.
### 5.3.3 Transmural heterogeneity in active mechanical properties

The force-stretch response of LMS from different layers of the LV wall was investigated by stretching them from 2.00 μm to 2.40 μm SL in 0.10 μm steps. We found that the midmyocardium produced significantly higher active force than the endocardium at 2.20-2.40 μm SLs (Figure 5.4A). Epicardial LMS were also significantly stronger than endocardial LMS at SL of 2.35 μm and in general trended towards higher force development across the entire spectrum of SLs examined (Figure 5.4A). The sensitivity of each layer to stretch was quantified by fitting a linear regression to the active force-SL data as outlined in 2.10.2. The gradient of this was significantly higher in epicardial compared to endocardial LMS (Figure 5.4B), meaning that for the same change in preload (i.e. SL) the epicardium undergoes a greater increase in its force output than the endocardium.

### 5.3.4 Transmural differences in cardiomyocyte size, morphology, and architecture

One early observation made with light macroscopy during this project was that LMS from the endocardium are patchier with intermittent gaps between myocardial fibres. Our next step was therefore to perform structural assessments and investigate whether there are differences in cellular morphology and extracellular tissue organisation, which could account for the differences in transmural mechanical behaviour.

Blinded immunohistochemistry quantification of cardiomyocyte area, morphology, and density was performed as detailed in 2.8 and 2.10.4 (Figure 5.5). Cardiomyocyte area has been suggested to be greater in rat endocardial than epicardial cardiomyocytes (Gerdes et al., 1986). There were no differences in cardiomyocyte area in LMS across the LV wall, however the total area covered by myocytes (cardiomyocyte area × cardiomyocyte density) was significantly larger in midmyocardial and epicardial compared to endocardial LMS (Figure 5.5B,D). There were no statistically significant differences in cardiomyocyte aspect ratio (Figure 5.5C).

Cardiomyocyte dispersion, a measure of the variability in cell direction from a main axis, was quantified by the standard deviation of the Gaussian curve fitted to confocal images as described in 5.2.6 and Figure 5.1. This is graphically demonstrated in Figure 5.5 by splitting each confocal image into 3 regions with an arrow representing the main cardiomyocyte direction of each region drawn. The higher the angle between the 3 arrows the higher the dispersion. This was significantly greater in epicardial compared to endocardial and midmyocardial LMS (Figure 5.5E).
Figure 5.4: Developed force and linear regression slopes obtained from Frank-Starling experiments

A) Active force-SL relationships of LMS from different layers of the LV wall. Linear regressions are fit to quantify the sensitivity of LMS to stretch. B) Slopes of linear regression compared with ANCOVA test. C) Representative twitch force transients of LMS at the same SL. N = 6. Data shown as mean ± SEM. Figure adapted from (Pitoulis et al., 2020) under CCL.
Figure 5.5 Transmural LMS cardiomyocyte heterogeneity

A) Representative images. B, C) Cardiomyocyte area, and aspect ratio (N = 180/5, 216/6, 216/6). D, E) Total myocyte area, and dispersion (N = 15/5, 18/6, 18/6). N = images or cells/replicates for endo-, mid-, and epicardial LMS. Data shown as median ± IQR for B, C and mean ± SEM for D, E. Adapted from (Pitoulis et al., 2020) under CCL.
5.3.5 Transmural passive mechanics and its molecular underpinnings

Passive force describes the resistance of the heart to stretch. The greater the increase in passive force per unit of stretch, the greater the ventricular resistance to blood filling. Passive force was significantly lower in endocardial compared midmyocardial, and epicardial LMS at SLs of 2.35- and 2.40 μm (Figure 5.6A). In contrast to our findings, the passive tension-SL relationship has been shown to be steeper in intact isolated cardiomyocytes from the endocardium compared to the epicardium (Cazorla, Le Guennec & White, 2000). Likewise, in skinned preparations the endocardium has a higher passive force than the endocardium at the upper SL range (Cazorla et al., 2005); yet others have suggested that no differences exist between the two (Haynes et al., 2014)(van der Velden et al., 2011) nor between them and the midmyocardium (Haynes et al., 2014). However, these data reflect the response of cardiomyocytes alone. We show that in intact living tissue, layers from the endocardium are less stiff than the epicardium and the midmyocardium.

Differences in the expression of sarcomeric proteins or ECM composition between layers could underlie these as well as the active force findings described in 5.3.3. We quantified the total sarcomeric protein and phosphoprotein status of these by performing Sypro Ruby and ProQ diamond staining on LMS (Figure 5.6B-D). Additionally, we quantified total collagen by performing transverse cryosections and picrosirius red staining to examine whether ECM composition shows heterogeneity across the LV wall (Figure 5.6E). No significant differences were found.
Figure 5.6: Passive force, sarcomeric protein to phosphoprotein ratios, and total collagen content

A) Passive force (N = 7, 5, 7). B, C, D, E) Ratio of phosphorylated to unphosphorylated myosin binding protein C (MyBP-C), troponin I (TnI), troponin C (TnC) and myosin light chain (MLC) (N = 4). F) % area of Sirius red staining (N = 6). N = endo-, mid- and epicardium. Data shown as mean ± SEM. Figure adapted from (Pitoulis et al., 2020) under CCL.
Figure 5.7: Sarcomeric protein and phosphoprotein as well as picrosirius red staining
A) Sarcomeric protein content (ProQ Diamond, left) and phosphorylated proteins (SYPRO ruby stain, right) of LMS from different layers of the LV wall. B) Picrosirius red staining of transverse sections from different layers of the ventricular wall. Figure adapted from (Pitoulis et al., 2020) under CCL.
5.4 Discussion

5.4.1 LMS from different layers of the LV wall show transmural mechanical heterogeneity

LMS from the midmyocardium and epicardium were stronger than endocardial LMS towards the higher spectrum of SLs examined. Additionally, epicardial LMS showed greater sensitivity to stretch as quantified by the linear regression lines. Importantly, these experiments were conducted whilst controlling for \([\text{Ca}^{2+}]_o\), ensuring that any differences reflected intrinsic mechanical differences.

Similar observations have been reported in permeabilised preparations from non-failing cardiac tissue, which show that the midmyocardium produces higher isometric force and power than the endocardium and epicardium (Haynes et al., 2014). Interestingly, this transmural difference is lost in samples from HF patients (Haynes et al., 2014). Whether the epicardium is stronger than the endocardium has been a subject of debate in the literature, and findings appear species-dependent. In intact isolated ventricular cardiomyocytes from ferrets the endocardium works on a steeper force-SL relationship than the epicardium producing greater force at the highest SLs (Cazorla, Le Guennec & White, 2000). However, no differences in force production between these layers were found in skinned pig myocytes (Stelzer et al., 2008) and, more comparably to our study, isolated rat cardiomyocytes (Cazorla, Le Guennec & White, 2000).

Generation of force depends on cardiomyocyte size, and density within a tissue. For example, in engineered heart tissues (EHTs), higher cardiomyocyte density corresponds with increased force generation (Weinberger, Mannhardt & Eschenhagen, 2017). We found that although cardiomyocyte area was not different, total myocyte area was significantly higher in epicardial and midmyocardial compared to endocardial LMS. Higher myocyte area means more active contractile units and could help explain the higher active force production as well as the steeper force-stretch response in mid- and epicardium. Additionally, a greater total myocyte surface area

Both midmyocardial and epicardial LMS were stronger than endocardial LMS, yet the midmyocardium produced greater active force than the epicardium and at a lower SL. Given that there were no differences in the total myocyte area between these two layers, we hypothesized that cardiomyocyte direction could explain the differences in force generation. Misalignment of cardiomyocytes can diminish force production (Munro et al., 2018) and greater dispersion was found in epicardial LMS. This could decrease force production and
provide an explanation as to why, despite their lower cardiomyocyte aspect ratio, they lag behind midmyocardial LMS.

To investigate the molecular profile of LMS from different layers we conducted quantification of total sarcomeric protein and phosphoprotein content as well as collagen. Sarcomeric proteins can impact both active and passive force, whilst uncoiling of collagen bundles at high diastolic stretch, accounts in part for the non-linear passive force-stretch relationship (de Tombe & ter Keurs, 2016). We did not find significant differences in these between layers. Similar homogeneity in the expression of sarcomeric proteins and their phosphorylation status have been demonstrated in human (Haynes et al., 2014) and rat (Campbell et al., 2013) hearts. Although transmural gradients in myosin light chain phosphorylation have been reported in the rat (Davis et al., 2001), we did not find this nor did others (Huang et al., 2008).

Titin, a giant sarcomeric protein that acts as a molecular spring, unfolding/refolding upon stretch, is another regulator of cardiac passive tension. Specifically, cardiac stiffness depends upon both titin content and isoform composition, and in the adult heart, titin exists as either N2BA (less stiff) or N2B (stiffer) isoform (Lewinter & Granzier, 2010). Although titin content or composition was not quantified here others have shown that in the dog and pig healthy heart the N2BA:N2B ratio is highest on the subendocardium (Bell et al., 2000). This would imply a more compliant endocardium, in accordance with our findings.

**5.4.2 Conceptual framework to fit transmural mechanical differences in cardiac muscle operation**

Data on *in vivo* SL are scarce, however reconstruction of SL across the LV wall using radiopaque beads and radiography show that the endocardium works at a higher and narrower SL spectrum compared to the midmyocardium and epicardium (Rodriguez et al., 1992). In fact, during end-diastole the former can attain 2.4 μm SL where most of the significant differences reported here arise, whereas the midmyocardium and epicardium work at 2.0-2.2 μm SL. Thus, differences in operating SL likely exist *in vivo*.

Furthermore, data from isolated arrested hearts show that deformation of the ventricular wall is not uniform (Omens, May & McCulloch, 1991). Transmural differences in normal strains exist and these increase in magnitude from outer to inner layers – that is, from epicardium to endocardium (Omens, May & McCulloch, 1991). We found that the SL-strain (% stretch) relationships are the same across LMS from different layers of the ventricular wall.
The result of a) uniform SL-strain relationships, but b) transmural strain gradients (highest on the inner LV wall) would allow the endocardium to reach its higher suggested \textit{in vivo} operating SL. This framework is supported by our findings of a more compliant endocardium that is less resistant to stretch. In this case, the greatest stiffness of the outer layers (midmyocardium, and epicardium) would enable them to act as ‘guardian’ layers, minimising excessive diastolic wall strain of the endocardium.

In addition to diastole, these findings are also relevant for systole. We found that the endocardium requires greater amount of stretch to produce the same amount of force as the outer layers. Given transmural and non-uniform operating SLs, the endocardium would be positioned up and to the right of the midmyocardium and epicardium in the Frank-Starling curves. This would offset intrinsic differences in contractility, enabling more homogeneous force generation and ventricular pumping.

\textbf{5.4.3 Limitations and future work}

The limitations of this study are discussed here. First, \textit{in vivo} the myocardium generates pressure and changes volume – that is, it operates in 3D. Here, LMS were stretched uniaxially in 2D along their dominant fibre direction. Secondly, we classified the 1\textsuperscript{st} & 2\textsuperscript{nd}, 3\textsuperscript{rd} & 4\textsuperscript{th}, and 5\textsuperscript{th} and 6\textsuperscript{th} generated LMS as subendocardium, midmyocardium, and epicardium. Although this enables comparison of transmural differences between the inside, mid, and outer wall, the distinction is arbitrary and ultimately an outcome of the method of LMS preparation. Thus, even though transmural differences exist, and we can quantify these, more reliable methods are needed to categorize LMS into endocardium, midmyocardium, and epicardium. Furthermore, as data from adjacent layers were collapsed for the endocardium, midmyocardium, and epicardium, the sensitivity may have been decreased and transmural differences underestimated.

All experiments shown here were conducted on LMS from healthy adult rats’ hearts. The presence of heterogeneous transmural profiles creates margins for these to be regionally disrupted, which could facilitate mechano-electrical mismatch across the LV wall and contribute to disease development. Proof of work has already shown that transmural differences may be decreased in the diseased myocardium and that this may have clinical consequences (Haynes \textit{et al.}, 2014). Future work leveraging LMS from samples of diseased myocardium, including human, could shed light into the mechanisms and magnitude of these changes.
Chapter Six

General conclusions
6 Overview

In this chapter the results of this PhD thesis are summarised in line with our original aims and objectives. The implications, strengths, and weaknesses of our findings are discussed, and future potential directions examined.

6.1 Recreating cardiac work loops in vitro

Mechanical load is instrumental for the normal operation of the heart. Although its importance for cardiac tissue culture has been established (Watson et al., 2019) (Vunjak Novakovic, Eschenhagen & Mummery, 2014) (Ronaldson-Bouchard et al., 2018), most contemporary culture protocols are oversimplified. The first aim of this project was the development of a platform to recreate the mechanical events of in vivo cardiac cycle in vitro.

In Chapter 3, we utilise a 3EWK mathematical model to mechanically load LMS and recreate cardiac work loops in vitro. Though the 3EWK has been used to acutely mechanically load cardiac tissue in the past (De Tombe & Little, 1994) (Garrett et al., 2019), to the best of our knowledge, this is the first time that it was used for long-term cardiac tissue culture. With this loading protocol, we were able to increase the culture duration of adult rat LMS to 72 hr, a threefold increase compared to the 24 hr culture stainless steel stretchers approach of (Watson et al., 2019).

Both preload and afterload are parametrised with our culture platform, enabling simulations of physiological but also pathological load (pressure- and volume-overload). This permits the study of the remodelling response to mechanical overload in vitro. We demonstrate (Chapter 3) that both pressure- and volume-overloaded LMS develop hypertrophic phenotypes compared to physiological load, yet functional remodelling is distinct with each load profile. Additionally, despite a common set of load-independent genes that undergo the change in direction of expression, most differentially regulated genes are unique and sensitive to the type of mechanical load applied to the LMS.

A noteworthy feature of our culture system was the negative feedback-based mode of operation of the 3EWK. For a given set of 3EWK parameters, the length-waveform applied to the LMS during culture would automatically adjust based on the force generated by the LMS. This was critical for afterload to be maintained constant and represents an adaptive solution to load cardiac tissue as it approximates the mechanics of the in vivo heart and its relation to the circulatory system. A different method to load the tissue using the 3EWK would involve maintaining CO constant by automatic and continuous adjustments of afterload. CO depends on SV and HR (equation (1)). For an LMS beating in vitro, SV is analogous to stroke length...
and HR is equal to the electrical stimulation frequency (kept constant during culture). In future studies, stroke length could be controlled (and by extension CO) by adjusting the 3EWK parameters using a force-feedback based protocol. This method would recreate a condition whereby the circulatory system is adapting and responding to the beating LMS (and not the other way around as is the case with constant afterload used in Chapter 3). The myocardial remodelling response to different CO could be investigated in this manner.

Baseline rat plasma concentrations of noradrenaline, adrenaline, triiodothyronine, and dexamethasone were added to the culture media to improve its proximity to blood. Although hormonal stimulation protocols have been used in the past (Yang et al., 2014b), this is the first time they have been applied to LMS culture. The concentrations were only added during media exchanges for two reasons: technical ease, and minimisation of number of interacting variables. In reality, hormones follow diurnal cycles and continuously interact with the cardiovascular and other organ systems. As such, our approach is distanced from the in vivo environment. However, it is much easier to add a standard hormone concentration than to constantly adjust it. Additionally, a constant hormonal level reflects a case of no interaction between hormonal and mechanical axis, which would have made subsequent interpretation of findings challenging. In the future, feedback-based perfusion systems that interact with a) the parameters inputted in the 3EWK model, and b) the force generated by the LMS, could be designed to adjust the neurohormonal concentrations continuously and automatically in the culture media. For example, if 3EWK parameters of pressure-overload are chosen, the concentration of sympathomimetic hormones could be simultaneously increased to increase LMS force generation. An increase in force reflects an increase in ΔP according to equation (2) and Laplace’s law (equation (3)), meaning that with such a system CO could be maintained constant against an increased TPR (Triposkiadis et al., 2009)(Kishi, 2012).

In summary, the work presented in Chapter 3 has permitted us to a) recreate cardiac work loops in vitro, b) parametrise preload and afterload, c) chronically maintain the myocardium with baseline hormonal levels, d) prolong culture to 72 hr, and e) simulate and study pathological remodelling in response to mechanical overload profiles in vitro.

Future work should focus on applying therapeutic modalities on the remodelled LMS to either prevent further deterioration or reverse the diseased phenotype. A first approach could involve the development and application of molecular tools (e.g. small interfering RNAs) on the LMS during culture. Pathways that could be targeted are already accessible and uncovered from the work conducted in this thesis (Chapter 3). An alternative therapeutic approach could involve mechanical unloading of the LMS to study reverse-remodelling. In its
simplest form, this would involve decreasing preload on the LMS after a predefined period of culture with pathological load. A more complex approach could incorporate resistive, inductive, and electrical potential generating elements within the 3EWK model to simulate the effects of left ventricular assist devices (Capoccia, 2015). Irrespective of therapeutic approach (molecular or mechanical), one critical question is the timepoint at which the therapy is applied. The myocardial phenotype observed in Chapter 3 was the outcome of 72 hr mechanical overload. As such, it would be sensible that all therapeutic modalities are applied to the LMS at this point and the total culture duration extended to 96 or 120 hrs.

Other labs have shown that culture of human LMS for up to 4 months is possible (Fischer et al., 2019), and although rat LMS are more sensitive to the culture environment, prolongation of culture to 120 hrs should be possible, particularly given the robustness of the LMS model at 3 days (Chapter 3).

6.2 Developing an automated bioreactor for scalable physiologically relevant LMS culture

Our findings from Chapter 3 demonstrated that with application of physiologically relevant mechanical load the LMS phenotype can be studied in both physiology and pathology for up to 72 hrs. However, the laboratory equipment used for these experiments had several limitations including a) cost, b) technically challenging to set-up and operate, c) not designed for culture experiments, and d) low throughput. The second aim of this project was to develop a culture bioreactor that would address the limitations of the Aurora set-up of Chapter 3 and increase the accessibility of LMS culture.

In Chapter 4, we demonstrate the development of MyoLoop, a bioreactor to mechanically load LMS using the 3EWK model. MyoLoop was designed for chronic in vitro culture, and except for the peristaltic pump it is self-sufficient. It automatically controls temperature, applies electrical field stimulation to the LMS, amplifies the LMS force signal, as well as automatically acquires, processes, saves & displays the force and length signals via the DAQ modules. Configuring the MyoLoop is considerably easier than the Aurora as the MyoLoop box is plug-and-play and almost all physical assembly parts (e.g. culture chamber, heater plate) are mounted using regular screws and nuts. Its user-interface is also simpler and requires no coding experience to install or operate. These features make the device more accessible, particularly to labs and researchers without coding, electronics, CAD, or machining experience. It is a scalable solution for in vitro experiments and a potentially promising device for basic cardiovascular research.
Despite development and validation of the device, we were unable to show an application. In Chapter 4, we performed drug toxicity studies by culturing adult rat LMS on MyoLoop for 72 hrs with or without 1 μM sunitinib. Although there were no differences, our findings were limited to functional studies; more thorough assessments of molecular and metabolic signatures are needed for conclusive results. Still, such experiments highlight the potential for MyoLoop to be used as a drug screening instrument. A number of further technological advancements are required for this to materialise. First, the system must be multiplexed. As detailed in Chapter 4, all the electronics on MyoLoop were designed to accommodate two systems – that is, two LMS can be run per MyoLoop box at the same time. However, due to time constraints, the software was written to acquire and send data (force and length respectively) to only one culture chamber. Thus, the first step in making MyoLoop a multiplexed device is to adjust the LabVIEW algorithms for dual and paralleled operation. This would permit stacking of MyoLoops in duplets. Secondly, the sensitivity of MyoLoop to detect the therapeutic or cardiotoxic effects of drugs must be assessed. This could be done by titrating across a range of doses of drugs with known consequences with subsequent mapping of functional, structural, and molecular effects. Then, MyoLoop could be used to uncover more subtle effects including the relationship between mechanical load, drug, and drug dose (Truitt et al., 2018).

Given interspecies differences, it is critical that drug experiments are conducted in the species of interest. Although all experiments shown in this thesis were on rat LMS, human LMS are a robust preparation that can be cultured for longer timeframes and has been used extensively by us (Watson et al., 2017)(Watson et al., 2019)(Camelliti et al., 2011)(Pitoulis et al., 2019a) and others (Qiao et al., 2018)(Fischer et al., 2019). The original 3EWK algorithms of the Aurora set-up were solved for the geometry of the rat LV, which prevented the use of human LMS. However, a module to permit selection between rat and human LV geometry for LMS culture was coded into MyoLoop. Coupled with the ability to pace tissue at different frequencies on MyoLoop (human LMS are typically cultured at 0.5- and not 1 Hz), this feature should be leveraged in future work to study human tissue.

Finally, MyoLoop could be used for EHT electromechanical stimulation and maturation protocols. Cardiac work loops are beginning to be incorporated in the maturation strategies (Ng et al., 2020)(Sewan, Shen & Campbell, 2021) of this field, yet they are currently limited to quasi-physiological methods without force-based feedback modules. Recreating the nominal cardiac work loop with force-feedback may be an essential component in generating tissue constructs with adult like features. This is further encouraged by the similarities of the
LMS culture and EHT maturation fields as protocols that work in one tend to work in the other.

### 6.3 Uncovering the transmural mechanical properties of the LV wall

Most projects in this thesis focused on long-term culture of LMS. However, multiple studies (Camelliti et al., 2011)(Pitoulis et al., 2019a)(Dries et al., 2021)(Wen et al., 2018) have demonstrated that LMS are highly reliable for acute experiments. In Chapter 5 we set out to investigate whether differences in mechanical properties of LMS prepared from the subendocardium, midmyocardium, and subepicardium exist, and if so the structural and molecular basis of these.

Although transmural studies using *ex vivo*, *in vitro*, and *in vivo* models have already been conducted; this was the first time that an intact *in vitro* model was used. Because of the nature of LMS preparation, different layers of the heart were split into discrete units while remaining intact. This had two major advantages: a) it enabled us to study the mechanical and molecular properties of each in isolation from the others such that intrinsic differences could be identified, and b) because the preparation was intact, functional studies on living tissue could be conducted. We found that mechanical heterogeneity exists between different layers of the LV wall. Our results may help explain the normal operation of the healthy heart.

In the past, (Haynes et al., 2014) have shown that in HF transmural mechanical heterogeneity is lost and that this may contribute to diminished prognosis, yet these findings were conducted on permeabilised cells. Future work using LMS is needed to uncover how these transmural relationships are impacted in diseased myocardial phenotypes. This is already happening; (Dries et al., 2021) used rat LMS to study the effects cryoinjury on the propagation and frequency of Ca$^{2+}$ waves and the endocardial vs. epicardial differences of this injury. Studies on pressure- or volume-overloaded phenotypes would also be insightful. These could be conducted by culturing LMS from different layers of the LV in MyoLoop and subsequently performing functional and molecular analysis. Alternatively, *in vivo* models of mechanical overload could be used, and the LV heart dissected at a later point. The temporal progression, if any, of these transmural relationships could be explored in this manner and implications for disease and prognosis pinpointed. Finally, like with culture experiments, more work is needed on samples from human tissue. Given the greater thickness of human tissue, it is likely that transmural differences, if present, will be accentuated.
6.4 Key messages of this work

We developed a platform to recreate the normal in vivo cardiac operation in vitro and show that the remodelling response of the heart to distinct mechanical load profiles can be chronically studied. We demonstrate the development of MyoLoop, a laboratory bioreactor that can be used to perform in vitro cardiac tissue culture experiments from mechanistic insight to toxicology and drug screening. Finally, leveraging the uniqueness of the in vitro model employed in this thesis we show that mechanical heterogeneity exists across the heart wall with implications for health and disease.
Chapter Seven

References


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Chapter Eight

Appendix
Appendix 1

#Imports

##General Imports
from Tkinter import*
import sys

##Stimulator imports
import stimulator_module

##Pump Imports
import pump_module

##Temperature Imports
import temperature_module_v4

#Initialise Tkinter
root = Tk()
root.title('MyoLoop')
# root.geometry('300x100')
degree_sign = u'N°

#Stimulator Options Menu
pulse = StringVar(root)
pulse_options = ['4 ms - 1Hz','8 ms -1 Hz','12 ms - 1 Hz','16 ms - 1 Hz','20 ms - 1 Hz', '4 ms - 0.5 Hz','8 ms - 0.5 Hz','12 ms - 0.5 Hz','16 ms - 0.5 Hz','20 ms - 0.5 Hz']
pulse.set(pulse_options[2])
pulse_menu = OptionMenu(root, pulse, *pulse_options)

#Start & Change Pulse Width
def set_pulse():
    stimulator_module.stop_wave()
    chosen_pulse = str(pulse.get())
    for p in pulse_options:
        if p == chosen_pulse:
            new_pulse = pulse_options.index(p)
            stimulator_module.start_wave(new_pulse)
    system_response.config(text = 'Stimulation changed to ' + str(pulse.get()) + '.')

#Stop Current Pulse
def stop_wave():
stimulator_module.stop_wave()

system_response.config(text = 'Stimulation OFF.')</n

#Pump
def get_and_set_rpm():
    try:
        rpm = int(set_rpm_entry.get())
        if rpm == 0 or (rpm > 50 and rpm <= 100): #Enter minimum value for pump
            system_response.config(text = 'RPM changed to ' + str(rpm) + '.')
pump_module.change_PWM(rpm)
        else:
            system_response.config(text = 'RPM must be 0 or between 50-100!')
    except ValueError:
        system_response.config(text = 'Please enter numeric values only for RPM n betwen 50 and 100 or 0.')</n

def pump_off():
    system_response.config(text = 'Pump OFF.')
pump_module.change_PWM(0)

#Temperature
desired_temperature = 36.0 #initial value
def set_temperature():
    global desired_temperature
    try:
        temperature = float(temperature_entry.get())
        if temperature >= 25 and temperature <= 38:
            desired_temperature = float(temperature_entry.get())
            system_response.config(text = 'Temperature set to ' + str(desired_temperature) + degree_sign + 'C.')
        elif temperature > 40:
            system_response.config(text = 'Temperature too HIGH!')
        elif temperature < 25:
            system_response.config(text = 'Temperature too LOW!')
    except ValueError:
        system_response.config(text = 'Please enter numeric values for temperature between 25 and 40.')
def temperature_iterator():
    global desired_temperature
    temperature_module_v4.supply_on(1)
    temperature_module_v4.temperature_main(desired_temperature)
data = temperature_module_v4.temperature_and_error_readout(desired_temperature)
    temperature_module_v4.supply_on(0)
    temperature_1 = data[0]
    temperature_2 = data[2]
    error_1 = data[1]
    error_2 = data[3]

    temperature_1_label.config(text = str(temperature_1) + degree_sign + 'C. ')
    temperature_2_label.config(text = str(temperature_2) + degree_sign + 'C. ')
    error_1_label.config(text = str(error_1))
    error_2_label.config(text = str(error_2))

    root.after(1000, temperature_iterator)

#Shutdown
def shutdown():
    stimulator_module.shutdown()
    pump_module.shutdown()
    temperature_module_v4.shutdown()
sys.exit(0)
    root.destroy()

#Create GUI buttons
##Stimulator
set_pulse = Button(root, text = 'Set New Pulse', command = set_pulse)
stop_button = Button(root, text = 'Stop Wave', command = stop_wave)
quit_button = Button(root, text = 'Quit & Clean', command = shutdown)
##Pump
set_rpm_entry = Entry(root, width = 10, borderwidth = 5)
get_rpm = Button(root, text = 'Set RPM', command = get_and_set_rpm)
turn_pump_off = Button(root, text = 'Pump OFF', command = pump_off)
##Temperature
temperature_entry = Entry(root, width = 10, borderwidth = 5)
temperature_entry.insert(0, 36.0)
temperature_1_label = Label(root, width = 5, borderwidth = 5)
temperature_2_label = Label(root, width = 5, borderwidth = 5)
error_1_label = Label(root, width = 5, borderwidth = 5)
error_2_label = Label(root, width = 5, borderwidth = 5)
set_temperature = Button(root, text = 'Set Temperature', command = set_temperature)

## General Text Box
system_response_label = Label(root, text = 'System:')
system_response = Label(root, borderwidth = 5)
system_response.config(text = 'Welcome to the MyoLoop GUI!')

# Pack GUI buttons

## Stimulator
set_pulse.grid(row = 0, column = 0, padx = 5, pady = 5)
stop_button.grid(row = 0, column = 1, padx = 3, pady = 5)
pulse_menu.grid(row = 1, columnspan = 2, padx = 5, pady = 5)

## Pump
set_rpm_entry.grid(row = 2, column = 0, padx = 5, pady = 5)
get_rpm.grid(row = 2, column = 1, padx = 3, pady = 5)
turn_pump_off.grid(row = 2, column = 2)

## Temperature
temperature_entry.grid(row = 3, column = 0)
temperature_1_label.grid(row = 3, column = 1)
temperature_2_label.grid(row = 3, column = 2)
error_1_label.grid(row = 4, column = 1)
error_2_label.grid(row = 4, column = 2)
set_temperature.grid(row = 3, column = 3)
error_general_label = Label(root, text = 'Temperature Error:')

## General
error_general_label.grid(row = 4, column = 0)
system_response_label.grid(row = 5, columnspan = 4)
system_response.grid(row = 6, columnspan = 4)
quit_button.grid(row = 7, columnspan = 4)

# Main
temperature_iterator()
root.protocol('WM_DELETE_WINDOW', shutdown)
root.mainloop()
Appendix 2

#Imports
import time
import sys
import spidev
import RPi.GPIO as GPIO
from numpy import log as ln

#Set-up ADC
CE = 0
spi = spidev.SpiDev()
spi.open(0,CE)
adc_channel_1 = 0
adc_channel_2 = 1
spi.max_speed_hz = 1000000

#Set-up GPIO
heater_1 = 15
heater_2 = 16
heater_3 = 32
heater_4 = 36
supply_1 = 13
supply_2 = 5
GPIO.setmode(GPIO.BOARD)
GPIO.setup(heater_1,GPIO.OUT)
GPIO.setup(heater_2,GPIO.OUT)
GPIO.setup(heater_3,GPIO.OUT)
GPIO.setup(heater_4,GPIO.OUT)
GPIO.setup(supply_1, GPIO.OUT)
GPIO.setup(supply_2, GPIO.OUT)

pwm_1 = GPIO.PWM(heater_1,1000)
pwm_2 = GPIO.PWM(heater_2,1000)
pwm_3 = GPIO.PWM(heater_3,1000)
pwm_4 = GPIO.PWM(heater_4,1000)
pwm_1.start(0)
pwm_2.start(0)
pwm_3.start(0)
pwm_4.start(0)

#Default Parameters
RT = 298.15
beta = 3976.0
nominal_resistance = 10000.0
Vin = 3.3

#ADC Functions
def ReadChannel(channel):
    adc = spi.xfer2([1,(8+channel)<<4,0])
    data = ((adc[1]&3) << 8) + adc[2]
    return data

#Apply PWM
def clamp(n, min = 0):
    if n < min:
        n = 0.0
    return n
def dutycycle(error):
    if error > 0.1:
        out = 100
    elif error <= 0.1:
        out = clamp(error/0.01)
    return out
# Convert ADC Result to Temperature

def convertToVout(ADCvalue, desired_temperature):
    Vout = ((ADCvalue*Vin)/1023)
    R2 = (Vout*nominal_resistance)/(3.3-Vout)
    temperature = round((1/(1/RT+(1/beta)*ln(R2/nominal_resistance))-273.15),1) # 2 decimals (number,2)

    error = round((desired_temperature-temperature),1) # 2 decimals (number,2)
    return (temperature, error)

def supply_on(value):
    GPIO.output(supply_1, value)
    GPIO.output(supply_2, value)

def temperature_main(user_temperature):
    data_1 = convertToVout(ReadChannel(adc_channel_1), user_temperature)
    current_temperature_1 = data_1[0]
    current_error_1 = data_1[1]

    data_2 = convertToVout(ReadChannel(adc_channel_2), user_temperature)
    current_temperature_2 = data_2[0]
    current_error_2 = data_2[1]

    pwm_1.ChangeDutyCycle(dutycycle(current_error_1))
    pwm_2.ChangeDutyCycle(dutycycle(current_error_1))
    pwm_3.ChangeDutyCycle(dutycycle(current_error_2))
    pwm_4.ChangeDutyCycle(dutycycle(current_error_2))

def temperature_and_error_readout(desired_temperature):
    data_1 = convertToVout(ReadChannel(adc_channel_1), desired_temperature)
    temp_1 = data_1[0]
    error_1 = data_1[1]

    data_2 = convertToVout(ReadChannel(adc_channel_2), desired_temperature)
temp_2 = data_2[0]
error_2 = data_2[1]
return (temp_1, error_1, temp_2, error_2)

def shutdown():
    spi.close()
pwm_1.stop()
pwm_2.stop()
pwm_3.stop()
pwm_4.stop()
GPIO.output(supply_1,0)
GPIO.output(supply_2,0)
GPIO.cleanup()
import pigpio

# Define Pins
source_pin = 5
sink_pin = 6
TTL_pin = 13

pi = pigpio.pi()

def create_waveform(waveform_list, total_active_ms, total_pulse_ms):
    for i in range(0, 11):
        if i%2 == 0:
            source_on = i*1000
            source_off = 2000
            sink_on = source_on
            sink_off = total_active_ms - source_on - source_off - sink_on - 5
            total = total_pulse_ms - sink_off
            pulse_features = []
            pulse_features.append(source_on)
            pulse_features.append(source_off)
            pulse_features.append(sink_on)
            pulse_features.append(sink_off)
            pulse_features.append(total)
            waveform_list.append(pulse_features)
        else:
            pass

# Frequencies
one_hertz = []
half_hertz = []
create_waveform(one_hertz, 810706, 1000000)
create_waveform(half_hertz, 1925300, 2000000)
# 1621800,378200)

def set_up():
    pi.set_mode(TTL_pin, pigpio.OUTPUT)
    pi.set_mode(source_pin, pigpio.OUTPUT)
    pi.set_mode(sink_pin, pigpio.OUTPUT)

    # 1Hz
    waveform_A1 = []
    waveform_B1 = []
    waveform_C1 = []
    waveform_D1 = []
    waveform_F1 = []

    # 0.5Hz
    waveform_A2 = []
    waveform_B2 = []
    waveform_C2 = []
    waveform_D2 = []
    waveform_F2 = []

    # #                              ON     OFF  DELAY
    waveform_A1.append(pigpio.pulse(1<<TTL_pin, 0, 5))
    waveform_A1.append(pigpio.pulse(1<<source_pin, 0, one_hertz[1][0])) #2ms ON
    waveform_A1.append(pigpio.pulse(0, 1<<source_pin, one_hertz[1][1])) #2ms OFF
    waveform_A1.append(pigpio.pulse(1<<sink_pin, 0, one_hertz[1][2])) #2ms ON sink
    waveform_A1.append(pigpio.pulse(0, 1<<sink_pin, one_hertz[1][3])) #turn sink off
    waveform_A1.append(pigpio.pulse(0, 1<<TTL_pin, one_hertz[1][4])) #finish pulse = 1000ms
waveform_B1.append(pigpio.pulse(1<<TTL_pin, 0, 10))
waveform_B1.append(pigpio.pulse(1<<source_pin, 0, one_hertz[2][0]))
waveform_B1.append(pigpio.pulse(0, 1<<source_pin, one_hertz[2][1]))
waveform_B1.append(pigpio.pulse(1<<sink_pin, 0, one_hertz[2][2]))
waveform_B1.append(pigpio.pulse(0, 1<<sink_pin, one_hertz[2][3]))
waveform_B1.append(pigpio.pulse(0, 1<<TTL_pin, one_hertz[2][4]))

waveform_C1.append(pigpio.pulse(1<<TTL_pin, 0, 10))
waveform_C1.append(pigpio.pulse(1<<source_pin, 0, one_hertz[3][0]))
waveform_C1.append(pigpio.pulse(0, 1<<source_pin, one_hertz[3][1]))
waveform_C1.append(pigpio.pulse(1<<sink_pin, 0, one_hertz[3][2]))
waveform_C1.append(pigpio.pulse(0, 1<<sink_pin, one_hertz[3][3]))
waveform_C1.append(pigpio.pulse(0, 1<<TTL_pin, one_hertz[3][4]))

waveform_D1.append(pigpio.pulse(1<<TTL_pin, 0, 10))
waveform_D1.append(pigpio.pulse(1<<source_pin, 0, one_hertz[4][0]))
waveform_D1.append(pigpio.pulse(0, 1<<source_pin, one_hertz[4][1]))
waveform_D1.append(pigpio.pulse(1<<sink_pin, 0, one_hertz[4][2]))
waveform_D1.append(pigpio.pulse(0, 1<<sink_pin, one_hertz[4][3]))
waveform_D1.append(pigpio.pulse(0, 1<<TTL_pin, one_hertz[4][4]))

waveform_F1.append(pigpio.pulse(1<<TTL_pin, 0, 10))
waveform_F1.append(pigpio.pulse(1<<source_pin, 0, one_hertz[5][0]))
waveform_F1.append(pigpio.pulse(0, 1<<source_pin, one_hertz[5][1]))
waveform_F1.append(pigpio.pulse(1<<sink_pin, 0, one_hertz[5][2]))
waveform_F1.append(pigpio.pulse(0, 1<<sink_pin, one_hertz[5][3]))
waveform_F1.append(pigpio.pulse(0, 1<<TTL_pin, one_hertz[5][4]))

## 0.5Hz
## 0.5 Hertz
waveform_A2.append(pigpio.pulse(1<<TTL_pin, 0, 5))
waveform_A2.append(pigpio.pulse(1<<source_pin, 0, half_hertz[1][0])) #2ms ON source
waveform_A2.append(pigpio.pulse(0, 1<<source_pin, half_hertz[1][1])) #2ms OFF
waveform_A2.append(pigpio.pulse(1<<sink_pin, 0, half_hertz[1][2])) #2ms ON sink
waveform_A2.append(pigpio.pulse(0, 1<<sink_pin, half_hertz[1][3])) #turn sink off and delay for a total TTL = 810.900 ms
waveform_A2.append(pigpio.pulse(0, 1<<TTL_pin, half_hertz[1][4])) #finish pulse = 1000ms

waveform_B2.append(pigpio.pulse(1<<TTL_pin, 0, 10))
waveform_B2.append(pigpio.pulse(1<<source_pin, 0, half_hertz[2][0]))
waveform_B2.append(pigpio.pulse(0, 1<<source_pin, half_hertz[2][1]))
waveform_B2.append(pigpio.pulse(1<<sink_pin, 0, half_hertz[2][2]))
waveform_B2.append(pigpio.pulse(0, 1<<sink_pin, half_hertz[2][3]))
waveform_B2.append(pigpio.pulse(0, 1<<TTL_pin, half_hertz[2][4]))

waveform_C2.append(pigpio.pulse(1<<TTL_pin, 0, 10))
waveform_C2.append(pigpio.pulse(1<<source_pin, 0, half_hertz[3][0]))
waveform_C2.append(pigpio.pulse(0, 1<<source_pin, half_hertz[3][1]))
waveform_C2.append(pigpio.pulse(1<<sink_pin, 0, half_hertz[3][2]))
waveform_C2.append(pigpio.pulse(0, 1<<sink_pin, half_hertz[3][3]))
waveform_C2.append(pigpio.pulse(0, 1<<TTL_pin, half_hertz[3][4]))

waveform_D2.append(pigpio.pulse(1<<TTL_pin, 0, 10))
waveform_D2.append(pigpio.pulse(1<<source_pin, 0, half_hertz[4][0]))
waveform_D2.append(pigpio.pulse(0, 1<<source_pin, half_hertz[4][1]))
waveform_D2.append(pigpio.pulse(1<<sink_pin, 0, half_hertz[4][2]))
waveform_D2.append(pigpio.pulse(0, 1<<sink_pin, half_hertz[4][3]))
waveform_D2.append(pigpio.pulse(0, 1<<TTL_pin, half_hertz[4][4]))

waveform_F2.append(pigpio.pulse(1<<TTL_pin, 0, 10))
waveform_F2.append(pigpio.pulse(1<<source_pin, 0, half_hertz[5][0]))
waveform_F2.append(pigpio.pulse(0, 1<<source_pin, half_hertz[5][1]))
waveform_F2.append(pigpio.pulse(1<<sink_pin, 0, half_hertz[5][2]))
waveform_F2.append(pigpio.pulse(0, 1<<sink_pin, half_hertz[5][3]))
waveform_F2.append(pigpio.pulse(0, 1<<TTL_pin, half_hertz[5][4]))

#clear waves
pi.wave_clear()

#add & create waves
pi.wave_add_generic(waveform_A1)
A1_wave = pi.wave_create()
pi.wave_add_generic(waveform_B1)
B1_wave = pi.wave_create()
pi.wave_add_generic(waveform_C1)
C1_wave = pi.wave_create()
pi.wave_add_generic(waveform_D1)
D1_wave = pi.wave_create()
pi.wave_add_generic(waveform_F1)
F1_wave = pi.wave_create()

pi.wave_add_generic(waveform_A2)
A2_wave = pi.wave_create()
pi.wave_add_generic(waveform_B2)
B2_wave = pi.wave_create()
pi.wave_add_generic(waveform_C2)
C2_wave = pi.wave_create()
pi.wave_add_generic(waveform_D2)
D2_wave = pi.wave_create()
pi.wave_add_generic(waveform_F2)
F2_wave = pi.wave_create()
all_waves = [A1_wave, B1_wave, C1_wave, D1_wave, F1_wave, A2_wave, B2_wave, C2_wave, D2_wave, F2_wave]
return all_waves

wave_list = set_up()

#start stimulator wave
def start_wave(pulse):
    pi.wave_send_using_mode(wave_list[pulse], 1)

#stop stimulator wave
def stop_wave():
    pi.write(TTL_pin, 0)
    pi.write(source_pin, 0)
    pi.write(sink_pin, 0)
    pi.wave_tx_stop()

#shutdown
def shutdown():
    pi.write(TTL_pin, 0)
    pi.write(source_pin, 0)
    pi.write(sink_pin, 0)
    pi.wave_tx_stop()
    pi.wave_clear()
    pi.stop()