Engineering the myocardial niche in a microscale self-assembling tissue-mimetic in vitro model

by

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Chemical Engineering and Applied Chemistry
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Abstract

Drug- and cell-based strategies for treating heart disease, including myocardial infarction, face significant roadblocks on the path to the clinic, a primary obstacle being the lack of information-rich in vitro human model systems. Conventional model systems are hampered by at least one of three fundamental limitations which include a) the lack of an in vivo-like microenvironment specifically engineered for the input cell population, b) a relatively low-throughput assays, and c) the low-content nature of output parameters. Herein we describe an integrated computational, design, and experimental strategy for the rational design of a microfabricated high-content screening platform which we term the Cardiac MicroWire (CMW) system. Within this system, we recapitulate the basic microenvironment found in the heart, one which integrates cardiomyocytes, non-myocytes, the extracellular matrix, and dynamic electromechanical forces. CMW are morphologically reproducible, maintain high cardiac sarcomeric protein expression in a highly dense, aligned, and suspended 3-D extracellular matrix, exhibit normal electrophysiological responsiveness, can be generated hundreds at a time. Application of this system to CMW composed of human Embryonic Stem Cell-derived cardiomyocytes under a rapid (7-day) maturation regimen yielded CMW with a conduction velocity of 47.4 ± 12.4 cm/s, on par with healthy human heart tissue. These results highlight the CMW system’s potential as a powerful discovery tool for screening small molecules and transplantable cells toward heart regeneration therapies.
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Introduction

1.1 Cardiovascular disease – myocardial infarction

An aging population and sedentary lifestyle have resulted in heart disease continuing to be a leading cause of death in the industrialized nations. Coronary heart disease, characterized by the accumulation of cholesterol-rich plaque within the coronary arterial wall (atherosclerosis), leads to arterial occlusion and the inadequate transportation of blood to and from the heart (myocardial ischemia). The lack of oxygen and nutrients delivered to the heart due to the blockage of blood vessels results in the onset of myocardial infarction (MI) or a heart attack. Tissue damage following MI occurs via an ischaemic cascade whereby affected areas of the heart muscle undergo metabolic waste buildup and cellular apoptosis. This is then followed by an inflammatory response which propels further death of cardiac muscle and surrounding tissues. In response, scar-forming myofibroblast cells begin to infiltrate the infarct area in an attempt at rapid provisional tissue repair. The newly formed scar tissue, however, is a composition of “patchwork” cell types which do not integrate functionally with the surrounding healthy myocardium. The fibrotic scar permits electrical conduction blocks in the normal electrophysiology of the heart along with biomechanical imperfections in the myocardium. These changes inhibit the heart’s ability to contract and pump blood efficiently, and can result in adverse effects to one or more of the stages in the normal cardiac cycle: diastole, systole, contraction, ejection, and relaxation. Heart condition continues to decline gradually and the onset of secondary diseases such as arrhythmias, hypertrophies, and further cardiomyopathies can begin to manifest and further the negative feedback loop of heart dysfunction.

1.2 Regenerative medicine

Regenerative medicine (RM) has established itself as a promising source of solutions to treating heart disease, however, many challenges remain. William A. Haseltine, in the year 1990s, made reference to the term ‘regenerative medicine’ to describe the integrated engineering of proteins, genes, and stem cells to repair diseased and damaged tissue (Graham, 2003). Since then, fueled
by advances of scientific technologies in materials science, protein and genetic engineering, developmental biology, computational modeling, and cellular biomechanics, among others, RM has evolved in parallel to describe the growing realms of biomedical engineering and biotechnological techniques used to repair, replace, maintain, and/or enhance organ function via 1) *in vivo* regeneration and remodeling of tissues by means of stimulating endogenous cells and 2) *in vitro* engineered tissue for a) transplantation and b) tissue modeling. *In vitro* tissue modeling is of great value in pharmaceuticals in the context of drug development tools – both for drug discovery and drug toxicity testing. It requires at least 10 years getting from the point of a drug discovery in the research laboratory through to clinical therapy in the hospital. Much of this time can be reduced with improved *in vitro* engineered tissue models, both in helping to choose preliminary drug candidates for a trial and with accelerating the assessment of the drug’s safety and efficacy.

## 1.3 Stem cells

Stem cells claim a central node to the ever-growing network of approaches in regenerative medicine. Self-renewal and differentiation potential are the two basic properties of stem cells, giving them the ability to occupy three fundamental states: expansion, maintenance, and exhaustion (see Supplementary Figure 1). Adult stem cells (aSC) can be found in many tissues of the body. During stages of organ growth and repair, these resident aSC are activated. The distinguishing characteristics between the various types of aSC in the body are their specific potencies for self-renewal and differentiation. For example, hematopoietic stem cells can give rise to and even replenish (via bone marrow transplantation) each of the blood cell types found in the circulatory system (Müller-sieburg et al., 2002). Cardiac stem cells, on the other hand, are comparatively limited and do not have an intrinsic proliferative potential to repopulate an injured region of the heart, let alone regenerate an entire heart (Laflamme & Murry, 2011; Segers & Lee, 2008). Human embryonic stem cells (hESCs), derived from the inner cell mass (ICM) of the preimplantation embryo, are pluripotent and thus have the ability to self-renew indefinitely and generate each of the cell types in the body, including germline cells and the extra-embryonic endoderm (ExE) lineages (Thomson, 1998). These unique and useful characteristics are what give hESCs their special focus and ever-growing interest in research, especially the efforts in
elucidating mechanisms involved in differentiating hESCs into specialized cell types. The discovery of human induced pluripotent stem cells (hiPSCs) (Takahashi et al., 2007), further prompts our need for creating robust, reproducible, and fully controllable methods of differentiating stem cells, with the intent of eventually scaling up towards clinical applications.

1.4 In vivo cardiac development

Following fertilization, the first steps of human embryogenesis produce the 16-cell morula via mitosis. The morula continues to divide and a cavity (blastocoel) develops on one side between the newly formed radial structure of cells (trophectoderm) and the inner cell mass (ICM); the structure is now identified as the blastocyst (Gilbert, 2000). The hypoblast, more commonly known as primitive endoderm (PrE), is the precursor in extra-embryonic endoderm (ExE), and first develops on the blastocoelic surface (the surface of the ICM exposed to the blastocoel). Through various cues delivered by the PrE, the ICM then develops into the primitive ectoderm, more commonly known as the epiblast, and the PrE differentiates into visceral endoderm (VE) and parietal endoderm (PE). A basal lamina layer also forms between the epiblast and the VE (Ungrin, Joshi, Nica, Bauwens, & Zandstra, 2008). PE and VE, once fully developed, play major roles in regulating mesendoderm differentiation. Gastrulation initiates early mesoderm specification via the emergence of the primitive streak. The expression of the T-box transcription factor Brachyury (Bry) represents the formation of the subset of cells responsible for the emergence and sustainment of the primitive streak. Cells in the streak undergo ingression and develop into definitive endoderm and mesoderm. The mesoderm germ layer is the precursor for hematopoietic, bone, and cardiovascular cells among others. Several genes including Bry, Wnt3a, Mixl1, FoxA2, Sox7, Sox17, GATA4, GATA6, and Nkx2.5 are expressed throughout this stage of specification and are often exploited in studies of early mesoderm induction (Celine L Bauwens et al., 2011; Elliott et al., 2011; Tran et al., 2009).

Structures of the heart begin to take shape on day 15 during cardiac morphogenesis. From a cluster of mesodermal cells without a defined architecture, the linear heart tube forms and begins to contract simultaneously during cardiac specification. The expanding primary heart field, also known as the cardiac crescent, is fed by the conjoined secondary heart field at three inlet points.
It is thought that the secondary heart field is a rich source of cardiac stem cells used for later development. Interestingly, the cells in the primary heart field will give rise only to the left ventricle; the rest of the heart is derived from the secondary heart field as it supplies cells into the primary heart field (Yeung, 2007). Symmetry of the structure breaks on day 21 as the entire heart tube loops and chambers begin to form. The left ventricle initiates and grows into a large spherical balloon derived from the primary heart field and the right ventricle initiates as an outgrowth anterior the left ventricle. As looping proceeds on day 28, the atria also balloon out and the positions of the chambers shift to allow the ventricles to be positioned anterior and inferior to the atria. The next three major stages of cardiac morphogenesis (up until day 56) consist mainly of the physical separation (septation) and growth of existing structures. Atrial septation, outflow tract septation, followed by ventricular septation allow for the heart to integrate and strengthen its existing architecture within itself and the circulatory system (Epstein, 2010). This is also a critical period of maturation for nodal and working-type cells in the heart chambers (Zhu et al., 2010).

1.5 In vitro cardiac differentiation

HESC isolation and in vitro differentiation has been an evolving art since it was originally done in 1998 by James Thomson (Thomson, 1998). There are many strategies currently used to induce the differentiation of hESCs. A conventional technique involves the use of human Embryoid Bodies (hEBs), which are aggregates of hESCs. Methods of generating hEBs include dissociated suspension culture (Doetschman, Eistetter, Katz, Schmidt, & Kemler, 1985), hanging drop culture (Dang, Kyba, Perlingeiro, Daley, & Zandstra, 2002; Hescheler et al., 1997), methylcellulose culture (Carpenedo, Sargent, & McDevitt, 2007; G. Keller, Kennedy, Papayannopoulou, & Wiles, 1993), spinner flask rendering (Cameron, Hu, & Kaufman, 2006; Dang & Zandstra, 2005), bioreactor culture (Niebruegge et al., 2009), and microwell technologies (Mohr, De Pablo, & Palecek, 2006; M. D. Ungrin et al., 2008). Upon the removal of the factors which maintain the pluripotent state, hEBs are capable of spontaneously differentiating into each of the three germ layers: endoderm, mesoderm, and ectoderm (Weitzer, 2006). Homogeneous differentiation of hEBs is a current technical challenge, and can start to be
solved through the improved spatial and temporal understanding of molecular signalling pathways active during early human development.

One of the main challenges of hEB-based hPSC differentiation is that heterogeneity and spatial disorganization leads to inefficient differentiation to specific cell types and a poor understanding of the mechanisms involved in lineage commitment. In studies addressing this challenge, two strategies have emerged. One involves controlling physical parameters of aggregate formation, such as aggregate size and shape (Céline Liu Bauwens et al., 2008; Burridge et al., 2007; Khademhosseini et al., 2006; Mohr et al., 2006; Ng, Davis, Azzola, Stanley, & Elefanty, 2005; Niebruegge et al., 2009; Pick, Azzola, Mossman, Stanley, & Elefanty, 2007; M. D. Ungrin et al., 2008). The second strategy focuses on exogenously controlling differentiation by delivering factors that are known or thought to be involved in specification, commitment, and proliferation of the cell type of interest (Laflamme et al., 2007; Nakanishi et al., 2009; Pick et al., 2007; Yang et al., 2008).

HEBs undergoing in vitro differentiation are thought to mimic the environment of the peri-implantation embryo where interactions between various cell types facilitate inductive events. Similar to the embryo, one of the earliest events during aggregate-based stem cell differentiation is the self-organization of a select population of cells into an outer epithelial layer of extraembryonic endoderm (ExE) cells surrounding an inner core of pluripotent cells (Abe et al., 1996; Coucouvanis & Martin, 1995, 1999; Grabel, Becker, Lock, Maye, & Zanders, 1998; G. M. Keller, 1995). Close proximity of precardiac mesoderm with these endodermal cells during embryonic development has been shown to play an inductive role. In exemplar, spontaneously contracting functional cardiac tissue was generated in cocultures of noncardiogenic embryonic tissue explants and endodermal tissue in vitro (Jacobson & Duncan, 1968; Nascone & Mercola, 1995; Orts Llorca, 1963; Schultheiss, Xydas, & Lassar, 1995; Sugi & Lough, 1994).

BMP and Activin A are synergistically involved in the induction of both precardiac mesoderm and definitive endoderm via the late stages of primitive streak formation (Yang et al., 2008). A recent paper by Kattman et al (Kattman et al., 2011) devoted a study to pinpointing the inductive effects of Activin and BMP signaling in cardiac differentiation of hESC in vitro. They reported that remarkably small variabilities in early exposure to Activin A and BMP concentrations can elicit significant changes in cardiogenic potential downstream. Specifically, there is a small
range of Activin A (3-6 ng/mL) and BMP (9-11 ng/mL) that result in an optimal cardiac induction efficiency for the system they studied. Even slightly larger concentrations of Activin A result in a drastic decrease of cardiac induction and increase in endoderm induction. Kattman et al go on to say that not only is there an optimal intermediate level of BMP4 and Activin A needed for efficient cardiac differentiation of the HES2 cell line (used in the study), but that there is an optimal level for each unique cell line, reinforcing the idea that not all hESC lines are comparable in their differentiation potential of the three germ layers and associated specialized cell types.

1.6 Micro-engineering the myocardial niche

The electromechanically dynamic properties of the heart are relatively complex and difficult to study at the macro level (Gurev, Lee, Constantino, Arevalo, & Trayanova, 2011). Microfabrication techniques have been employed to isolate many of these cell–cell and cell–ECM interactions to better understand how they affect the tissue remodelling capabilities of the various cell types found in the heart (Murtuza, Nichol, & Khademhosseini, 2009). Both direct and indirect techniques have been advanced in the field which allow for exquisite control of mechanical stimulation and measurement parameters (Iribe, Helmes, & Kohl, 2007; Moraes, Chen, Sun, & Simmons, 2010; Moraes, Wang, Sun, & Simmons, 2010; Saenz Cogollo, Tedesco, Martinoia, & Raiteri, 2011; Yin et al., 2005). Development of high-fidelity microenvironments that accurately represent the in vivo niches of cells in their native environment, such as substrate stiffness (Bhana et al., 2010; Jacot, McCulloch, & Omens, 2008), substrate topography (D.-H. Kim et al., 2010), localized concentrations of growth factors (Kattman et al., 2011), and electrical stimulation (Dengler et al., 2011), among others, will enhance our understanding of the mechanotransduction biology in the heart.

Generating a physiologically accurate cardiac in vitro model is challenging. Much of the effort in interrogating and recapitulating the in vivo environment has been focused on the strains and stresses (post-diastolic expansion and post-systolic contraction respectively) encountered by cardiac myocytes and fibroblasts alike in the mechanically dynamic environment of the heart (Curtis & Russell, 2011). Two-dimensional monolayers have been largely used as a platform for
studying the mechanical interactions of cells and their matrices (Kapoor, Caporali, Kenis, & Stewart, 2010; Liu et al., 2010). Although 2D models offer practicalities in experimental methods including the ease of isolation of different parameters, these models do not accurately mimic the physiology of the microenvironment of interest.

A primary obstacle with more realistic 3D models, however, is the diffusion limitation that is associated with thick tissues, both for oxygen and nutrient exchange (Radisic, Deen, Langer, & Vunjak-Novakovic, 2005) as well as for antibody-based immunofluorescence imaging. Furthermore, the added dimension in a 3D tissue brings with it complexity in the analysis of cell–cell and cell–ECM interactions. Miniaturizing 3D tissues to the sub-300 μm scale to eliminate diffusion barriers greatly simplify technical obstacles. Microtissues can also be generated with low volume components, and can be engineered to be highly controlled and reproducible. Hydrogel-based microtissues that self-assemble under gel compaction are a simple-to-generate system that is widely used for multiple cell types and matrices (Bell, Ivarsson, & Merrill, 1979; Brien, Zegers, & Mostov, 2002; Stopak & Harris, 1982).

A study by Legant et al makes use of this technique alongside a microfabricated device to both measure and manipulate forces exerted by micro-scale tissues (Legant et al., 2009). The study employed rectangular micro-wells containing two microcantilevers which simultaneously constrained the remodelling of the collagen with embedded 3T3 fibroblasts and reported real-time dynamic forces exerted by the contained microtissue. Similar cantilever-based force sensors have also been employed for studying the traction force of single cells (Fu et al., 2010). The study goes on to show that forces exerted by the cells (contractility), increased with tissue matrix and boundary rigidity, whereas protein expression associated with ECM and cytoskeleton correlated with mechanical stress. These types of microsystems are geared towards drug screens; compared to typical 2D culture systems used for screening purposes, these systems provide in vivo like organoid morphology and can serve as a validation to preliminary hits.

Tissues for eventual organ repair can also be engineered on a larger scale still employing microfabrication methods to achieve physiological properties. Bian et al have developed a method to generate aligned collagen-based tissue using a bed of macropillars (Bian, Liau, Badie, & Bursac, 2009). The technique takes advantage of gel compaction around an array of 2 mm tall
pillars; the geometry and spacing of the pillars are strategically designed such that the surrounding collagen remodelling results in tension-mediated alignment.

Controlling cell assembly and tissue formation are not only achieved through the micro- and nanofabrication approach, instead methods such as mechanical (Black, Meyers, Weinbaum, Shvelidze, & Tranquillo, 2009; Zimmermann et al., 2000) and electrical stimulation (Radisic et al., 2004) also play a critical role in building a functional cardiac tissue. Both mechanical and electrical stimulation have been shown to induce cell alignment in both 2D and 3D tissue constructs. In addition, both have been shown to induce cell maturation, ultrastructural organization and improve functional properties of the tissue such as contractile force. These methods are discussed in detail in other review articles (Iyer, Chiu, Reis, & Radisic, 2011). The combination of electrical pacing, mechanical stimulation and micro/nano-scale control of substrate topography is expected to significantly improve functional assembly of cardiac tissues in vitro.

1.7 Applications in advanced drug screening

Cell-based in vitro testing is most often the first step in the process of screening drug candidates for efficacy and potential toxicities. These in vitro screening platforms need to accurately replicate the response of in vivo pharmacology and toxicology in order to be an efficient and valuable preliminary tool. The challenge remains to be able to strategically balance the luxury of high-throughput systems with model complexity. Model complexity allows for the correct cell-cell, cell-ECM, and finally, cell-drug interactions to take place which will then provide the user with a correct in vivo-like response to the input drug. Additionally, the information-rich output parameters must also be easily accessible. The system which we are proposing aims to be an intermediary organotypic system with the capability to be screened with hundreds of factors (instead of thousands), as well as the ability to provide high-content readouts. A preliminary screen must be done to determine a shortlist of a few hundred factors, either through a conventional monolayer in vitro model-based screen, or computation method-based screen to predict potentially effective factors in isolation or in combination. Another potential source of promising drugs are those that have been discarded due to toxicity and/or inefficacy. If we can
make minor modifications to these drugs and re-evaluate them in our high-content system which is a more reliable indicator of potential, then these discarded drugs can be repurposed for clinical trials. Our system, which provides not only protein expression outputs, but also functional outputs, will indicate a panel of parameters on which the user can confidently evaluate the efficacy and toxicity of the input factor(s) for further in vivo testing. Although the current system is not designed to measure three key functionality outputs (force of contraction, fractional shortening, and electrical activity (via electrodes) – which includes AP duration, QT interval, and rate), these will be integrated within the next iterations of the design.

1.8 Rationale and objectives

High content screens using cardiac microtissue-based in vitro models, which closely mimic native heart tissue, can be used as an increasingly accurate tool for validating hits determined by preliminary screening techniques. We propose the design and implementation of a fully defined micro-scale model system, which we term Cardiac MicroWire (CMW), composed of human Embryonic Stem Cell-derived Cardiomyocytes (hESC-CM) in a hydrogel substrate that can be driven by an external pacemaker. Output capabilities of the system will include cell and tissue morphology, marker expression via whole-mount immunofluorescence, electrophysiological data, and gene expression.
2 Hypothesis

Precise microengineering of the human heart cell niche in a microtissue model can be used to interrogate hPSC-derived cardiac tissue phenotype and functionality. Miniaturization can enable higher throughput platforms and greater reproducibility of tissue generation and response, while retaining fundamental characteristics of three-dimensional myocardium.

3 Specific aims

3.1 Aim 1

Determine a rational design for a high-content three-dimensional cardiac microtissue array platform which recapitulates key aspects of the myocardial niche.

3.2 Aim 2

Characterize cardiac microtissue morphology (sarcomeric protein expression, cell elongation and alignment) and physiology (excitation threshold, maximum capture rate, and conduction velocity) in situ.
4 Materials and methods

4.1 Atomic force microscopy of collagen deposited slides

The data were collected on a Nanoscope IIIA Bioscope AFM. Tapping mode imaging was used in air using a TESP cantilever at a drive frequency of ~320 Khz, at a scan rate of 1 Hz (as a 512 x 512 pixel image) using the Nanoscope software version 5.30A.

4.2 Preparation and seeding of collagen deposited slides

Samples from collaborators at FibrAling Corporation are received loaded into tubes from with an uncoated glass border at the grasping end. Using clean forceps/tweezers, the glass chips are removed by their top grasping edge. Using the light reflectance to choose the collagen coated side, glass chips are placed in sterile tissue culture plates making note that fibril (coating) direction is parallel to collagen-glass border (as shown in Figure 1).

Glass chips are dipped into Dulbecco's Phosphate Buffered Saline (DPBS) for 20 seconds (Sigma D8537). They are then immediately rinsed in DI water using a gentle rotation motion for 5-10 seconds. They are then gently blow dried with clean, dry nitrogen or air and then immersed in 70% ethanol for a minimum of 1 hour to sterilize. After rinsing the sample in DPBS, slides are allowed to sit in culture media for 15 minutes before being seeded with cells. Cells are

Figure 1. Collagen deposited glass chip. Collagen fibrils are aligned perpendicular to the etch mark.
suspended in culture media at desired density and pipetted onto slide in a tissue culture plate. The cells are given 2 days to adhere before exchanging culture with fresh media.

4.3 Isolation of rat neonatal cardiomyocytes

Rat neonatal cardiomyocytes were isolated as previously reported (Dengler et al., 2011). Briefly, hearts were isolated from 1- to 2-day old neonatal Sprague Dawley rats using protocol approved by the University of Toronto Committee on Animal Care. Rat hearts of 1 to 2 litters of approximately 13 pups/litter were aseptically excised and placed in cold Hanks balanced salt solution (HBSS, Sigma), washed several times with HBSS and quartered (see Figure 2). Quartered hearts were then incubated overnight at 4 °C in a 0.06% w/v solution of Trypsin (Gibco) in HBSS on an orbital shaker at 50 RPM (Labent Orbit LS, Mandel). After 14-16 hours, hearts were washed with CM culture medium (high glucose [4.5 g/L] Dulbecco’s Modified Eagle Medium (DMEM) with L-glutamine (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% penicillin/streptomycin (Gibco) and 1% N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES, Gibco)) and subjected to a series of five digests (8 minutes, 37°C, 70 rpm) in a 0.1% (w/v) solution of collagenase type II (Worthington) in HBSS. The supernatant of each digest was collected, centrifuged (5 minutes, 750 rpm) and resuspended in CM medium. Cells were pre-plated for 60 minutes on tissue culture polystyrene (TCP) T75 flasks (BD Flacon) to enrich for cardiomyocytes (non-adherent cells). The supernatant was collected, and cell number was determined via trypan blue (Gibco) exclusion.
Cardiac differentiation of human Embryonic Stem Cells was carried out as reported previously (Celine L Bauwens et al., 2011). In this study, the HES2 (ES Cell International) hESC line was used. The hESC were maintained and expanded as described previously (Celine L Bauwens et al., 2011; Céline Liu Bauwens et al., 2008; L. Yang et al., 2008). Briefly, HES2 cells were passaged (up to 5 times) on mouse embryonic feeders (MEF) for 6 days in HES2 maintenance media (80% DMEM/F12, 20% KOSR, 20 ng/mL bFGF, 0.5% P/S, 1% NEAA, 1% BME), media was changed daily. Cells are maintained in normoxia at 37°C in a 5.0% CO₂ atmosphere. The cells were then trypsinized along with MEFs and plated onto Matrigel (diluted at 1:30) coated plates at a split ratio of 1:3 for MEF depletion. After two days of MEF depletion, HES2 cells were again trypsinized and seeded into AggreWells manufactured in-house to form hEB.

**4.4 Cardiac differentiation of human Embryonic Stem Cells**

Cardiac differentiation of human Embryonic Stem Cells was carried out as reported previously (Celine L Bauwens et al., 2011). In this study, the HES2 (ES Cell International) hESC line was used. The hESC were maintained and expanded as described previously (Celine L Bauwens et al., 2011; Céline Liu Bauwens et al., 2008; L. Yang et al., 2008). Briefly, HES2 cells were passaged (up to 5 times) on mouse embryonic feeders (MEF) for 6 days in HES2 maintenance media (80% DMEM/F12, 20% KOSR, 20 ng/mL bFGF, 0.5% P/S, 1% NEAA, 1% BME), media was changed daily. Cells are maintained in normoxia at 37°C in a 5.0% CO₂ atmosphere. The cells were then trypsinized along with MEFs and plated onto Matrigel (diluted at 1:30) coated plates at a split ratio of 1:3 for MEF depletion. After two days of MEF depletion, HES2 cells were again trypsinized and seeded into AggreWells manufactured in-house to form hEB.

**Figure 2. Cell sourcing cardiomyocytes from neonatal rat hearts.** Primary cardiomyocytes are isolated from 2 day old Sprague Dawley neonatal rats. Isolation consists of an overnight Trypsinization step and Collagenase cycles to dissociate heart to single cells. The final population consists of cardiomyocytes (CM), endothelial cells (EC), and cardiac fibroblasts (FB) after pre-plating steps to remove adherent FB and increase non-adherent CM percentage.
hEB were generated using 400µm microwell PDMS inserts cast from a silicon master mould. The inserts were cut and glued into 24-well tissue culture plates and then sterilized using ethanol. The microwells were then coated with 5% Pluronic Acid for at least an hour and washed with PBS before cell seeding. A single cell suspension of aggregation media containing base media and T0 cytokines (see Figure 2) supplemented with ROCK inhibitor Y-27632 was then seeded into the wells and allowed to aggregate overnight after centrifuging at 200 g. Cells are maintained in hypoxia at 37°C in a 5.0% CO₂ and 5.0% O₂ atmosphere. After 24 hours, hEB were formed and aggregation media was exchanged for T1 media. On day 4, hEB were removed from AggreWells and placed in Low cluster 6-well plates (NUNC). Corresponding media for T4, T8, T12 was freshly made and exchanged. On T12, cells were returned to normoxia at 37°C in a 5.0% CO₂ atmosphere. Media was replaced every 8 days onward.

![Figure 3](image_url)

**Figure 3. Cell sourcing human cardiomyocytes via cardiac differentiation of hESC.** HESC are differentiated using our AggreWell system for 20 days as Embryoid Bodies (EB) through three stages: mesoderm induction, cardiac specification, and proliferation. Each stage requires a specific induction media with a set of growth factors.
4.5 Microfabrication

Masks for master patterning were designed using AutoCAD (AutoDesk, San Rafael, CA, USA) and were printed at a resolution of 20000 dpi (CAD/Art Services, Bandon, OR, USA). Microfluidic cell culture devices were fabricated at the Emerging Communications Technology Institute cleanroom at the University of Toronto. Briefly, piranha washed 3x5” clean glass slides (Corning Inc., Corning, NY, USA) were given a brief wash in acetone and blow dried under a clean stream of Nitrogen gas. A seed layer of SU-8-5 (Microchem, Newton, MA, USA) (7 um high) was spin-coated on the surface to allow for feature layer bonding. Following a dehydration bake at 20 min on a 100 degrees Celsius hot plate, the slides were then cooled to 65 degrees Celsius and then removed from the hot plate to return to room temperature. UV expose the seed layer and initiate post-bake as before. Take slides with seed layers and apply 2 spin coat layers SU-8-50 (Microchem) sequentially (including pre- and post-bakes) to reach a feature layer height of 300 um. UV expose with designed mask onto master with 300um high feature layer. Post bake allow sufficient time to cool. Immerse in Developer (Microchem) on a sonicator or orbital shaker until un-crosslinked SU-8 is washed away thoroughly. Masters were oven-baked for 3 days at 75 degrees Celsius to allow proper bonding of feature layer to glass slide. Silanize masters in desiccator overnight. Primary replicates were manufactured by molding poly(dimethylsiloxane) (PDMS, Dow Corning) on SU-8 masters at 65 degrees Celsius overnight. Replicated were modified under a stereomicroscope and a negative master was molded using polyurethane (SmoothCast). Final substrates were then PDMS molded form these negative masters and outfitted into a 24-well tissue culture plate.

4.6 Generation, cultivation, and imaging of cardiac microtissues

Either rat neonatal CM or hESC-derived CM suspended in a collagen mastermix and seeded into cardiac microtissue wells at a density of 0.5 x 10^6 cell/mL. Microwell substrates were prepared by sterilizing with ethanol, washing and coating with 5% Pluronic Acid for at least an hour each. While coating, rat neonatal CM and/or hESC-derived CM are prepared. Aggregates from hESC-CM differentiation are put in Collagenase for 1 hour with DNAse in the incubator. Aggregates are then immersed in 0.25% Trypsin for 5-10 minutes with DNAse. Aggregates are then
immersed in STOP solution (50% FBS and 50% DMEM F12) and triturated with a 20-gauge syringe 10 times. Once aggregates are single cells, they are immersed in STAIN solution (10% FBS and 90% DMEM F12) and counted. The collagen mastermix is prepared by combining the following: 10X M199 (GIBCO), Glutamax (GIBCO), Collagen 1 (3.66 mg/mL) (BD), Glucose (0.3 g/mL) (GIBCO), NaOH (SIGMA), NaHCO3 (0.075 g/mL) (SIGMA), Heps (GIBCO), GFR Matrigel (BD), ddH2O at appropriate ratios for desired collagen concentrations. The collagen mastermix is constantly kept on ice under 4 degrees Celsius to prevent premature crosslinking. Finally, pipet 500 uL of mastermix into each well (of 24-well plate) and centrifuge at high speed (300 g) to eliminate bubbles. Maintain centrifuge at ice-cold temperature. Prepare cell-laden collagen (additional 250 uL per well) and pipet/mix into well to bring final cell density to 500,000 cells per well (final volume in each well should be 750 uL). Centrifuge entire plate (200 g) to force cells into microwell recessions. Carefully and slowly aspirate excess cell-laden collagen in each well to leave pockets of cell-laden collagen in each microwell. Place entire plate into normoxic incubator for 15 minutes. After 15 minutes, add 1 mL of cell culture media slowly as to not disrupt the polymerized collagen microtissues. Exchange media every 4 days. Microtissues should remodel between 1-3 days depending on input cell composition. Imaging of microtissues can be done in-situ. Samples can also be fixed, permeablized, and stained inside the microwells and imaged using a fluorescence microscope.

4.7 Electrical stimulation and functional analysis

For electrical point stimulation, microwells were embedded with 0.005” platinum wires (99.99% purity, A-M Systems Inc.) and hooked up to a commercial stimulator (Model S88X Grass, AstroMed® Inc.). After 72 hours of cultivation without electrical stimulation, the microtissues to be stimulated were stimulated with biphasic, square pulses, 1ms in duration, threshold amplitude of 6V (field strength of 6 V/cm) and frequency of 1Hz for the remainder of cultivation (4 days). The stimulation voltage was selected to induce synchronous construct contractions. Constructs were held in place within the PDMS substrate. 0.1mm stainless steel Minutiens pins (Austerlitz) were used to ensure the microtissues did not slip out. There is a considerable amount of difference in terms of what the cells undergo when stimulated through a field versus a point. In our system, the stimulation is done via point stimulation with platinum wire electrodes which are
more physiologic compared to field stimulation (stimulation provided by the Purkinje Fibres are more comparable to point stimulation rather than field stimulation). Both carbon and platinum electrodes have been shown to be advantageous for long term pacing with minimal toxicity effects to cells in culture.

Tissue function was established by measuring excitation threshold (ET), the minimum voltage required to pace the tissue simultaneously, and maximum capture rate (MCR), the maximum stimulation rate at which the construct can be induced to beat simultaneously, at 7 days after cell seeding. Tissue constructs or CM aggregates were individually placed between a pair of carbon electrodes in stimulation chambers (autoclaved before use). ET (V/cm) was measured by stimulating the tissue with square pulses of 2ms pulse width at a frequency of 1 or 2 Hz and gradually increasing the output voltage of the stimulator until >80% of the tissue was beating synchronously with the stimulator output. MCR was measured by setting the output voltage at 12 V, and increasing frequency until >80% of the tissue was no longer synchronously beating with the driving signal. All measurements were taken using an Olympus 1X2-UCB inverted fluorescent microscope housed in an environmental chamber (SolentScientific) maintained at a temperature of 37°C, and equipped with a Retiga camera (QImaging).

4.8 Flow cytometry

Aggregates are dissociated using collagenase treatment and Trypsin and immediately fixed with 4% paraformaldehyde (PFA) overnight at 4 degrees Celsius. They are then permeabilized at room temperature with 100% methanol for 2 minutes. Primary antibody is added after a 2% HF wash. It is then incubated at room temperature for 20 minutes. Next, the sample is washed with HF, and secondary antibody is added for another 20 minutes at room temperature. Lastly, the sample is washed again and is ready for flow cytometry analysis. The samples were always kept on ice before measuring on the flow cytometer.
4.9 Immunostaining and image analysis

Microtissues are washed with PBS and fixed for 24 hours with 4% PFA at 4 degrees Celsius. They are then permeabilized in 0.1% Triton X in blocking solution (Normal Donkey Serum). Primary antibody is then added for 3 days at 4 degrees Celsius. Lastly, the microtissues are washed three times and stained with the appropriate secondary antibody (AlexaFluor series) and with DAPI for nuclear staining, for one day overnight in the fridge. Each incubation step is preferably performed on a rocker table. Before imaging, the sample was washed three times and resuspended in 2% HF. Samples were imaged using a confocal microscopy (FV1000 laser scanning confocal; Olympus). All image analysis was done using custom macros built in ImageJ (cell alignment and elongation analysis, and total cell marker expression enumeration).

4.10 Optical mapping

For optical measurements, microtissues were stained with 5mM of Di-4-ANEPPS (Invitrogen, Carlsbad California) voltage-sensitive dye for 20 minutes, followed by 3 washouts with fresh warm Tyrode’s solution (Sigma-Aldrich) adjusted to pH 7.4. The temperature was kept constant at 37°C using a block incubator. Dye fluorescence was recorded using a microscope mapping system (Ultima, Scimedia, Tokyo Japan). The system included a CMOS camera with a 1cm sensor (100x100 pixel) attached to a custom-built microscope using PLAN APO objective and condensing lenses (Leica Microsystems GmbH, Wetzlar Germany), giving a magnification of 1.5X. The spatial resolution was 63μm/pixel. The fluorescence was excited using a Xenon light source (Moritek Corp. Japan) and a 530nm green filter (Semrock, Rochester NY) and the emission signal was long pass filtered using a 610nm red filter. Tissue constructs were point stimulated at 1000-ms cycle length using a bipolar electrode made with 2 fine silver wires (AWG#32) inserted in a large stainless steel needle mounted on a micromanipulator. Spontaneous tissue beating was also recorded, in addition to responses to frequency sweep from 1 to 5 Hz. Local activation times were measured at the peak of dF/dT for each pixel. Activation maps were constructed for a selected beat. Conduction velocity was calculated at each location using activation times of 9 neighbouring sites. Conduction velocity values from all sites were used to calculate the average conduction velocity across the construct surface; minimum and
maximum values were also noted. Phase contrast images of microtissue surfaces were taken prior to optical mapping, to correlate tissue architecture geometry with conduction velocity.

4.11 Computational modeling

The evolution and contractility of sarcomeric filaments in the microtissues is simulated using a framework proposed by Deshpande et al (2006). While this framework has previously been implemented for the modelling of stress fibres contractility in a range of cell phenotypes (Deshpande et al., 2007; Pathak et al., 2008; McGarry et al., 2009; Legant et al., 2009), here it is adapted for the simulation of sarcomeric filaments in cardiomyocytes on the basis that both stress fibres and sarcomeric filaments are composed of and operate via actin-myosin interactions. Briefly, the microtissue is modelled as a continuum in which sarcomeric filaments are free to form in all directions at all point in the tissue. The non-dimensional sarcomere activation level, $\eta(\phi)$, is computed in all directions ($\phi$). A first order kinetic equation governs the evolution of sarcomeric filaments, whereby filament formation is driven by a signal that decays exponentially with time (first term on the RHS of equation 1).

$$\dot{\eta}(\phi) = \left[1 - \eta(\phi)\right] \frac{c_\eta}{\theta} - \left(1 - \frac{\sigma(\phi)}{\sigma_0(\phi)}\right) \eta(\phi) \frac{k_\eta}{\theta}$$ (1)

Sarcomere contractility is modelled using a Hill-type equation, whereby the tension generated by a sarcomeric filament decreases with increasing shortening velocity. Hence, as sarcomeric filaments shorten, a reduction in tension occurs. Such a reduction in tension below the isometric value leads to partial dissociation of the sarcomeric filament, as captured by the second term on the RHS of equation 1.

Finite element (FE) models are created for both the “biaxial” and “uniaxial” microtissue, as shown in Figure C1. Undeformed FE geometries are based on initial microtissue geometries prior to deformation due to cardiomyocyte contractility and remodelling. The circular PDMS posts used to constrain the microtissues are modelled as rigid surfaces, as these supports are several orders stiffer than the surrounding microtissue. In the case of the biaxial FE model, the microtissue is assumed to be bonded to the eight supporting circular posts, reflecting the in-vitro low-concentration coating of each post with Pluronic Acid F-127 (a tissue adhesion-inhibiting
agent). In the case of the uniaxial microtissue, a higher concentration of Pluronic Acid F-127 was used, hence in the uniaxial FE models hard contact is assumed between the two circular rigid posts and surrounding microtissue, allowing sliding and separation of the tissue from the posts. 4241 plane stress full integration elements were used for the uniaxial geometry while 37603 such elements were used for the biaxial geometry for all analyses following an initial mesh sensitivity study.

### 4.11.1 Output parameters

The formation of aligned contractile sarcomeres at each point in the tissue is predicted by the output parameter $\Pi$, defined as the difference between maximum and mean sarcomere activation level ($\Pi = \eta_{max} - \bar{\eta}$). Highly activated, aligned sarcomere formation in a dominant direction is predicted by a value of $\Pi$ close to 1. In contrast, a value of $\Pi$ close to 0 predicts that no dominant sarcomere has formed at that point. Predicted distributions of $\Pi$ are directly comparable with fluorescent microscopy images stained for cardiac Troponin T and Alpha-Actinin, whereby removal of background fluorescence reveals the distribution of dominant sarcomere formation.

In order to investigate the relationship between sarcomere formation and the stress state of the tissue, a non-dimensional effective stress $\tilde{\sigma} = (\sigma_{max}^p - \sigma_{min}^p)/\sigma_{max}^p$ is defined where $\sigma_{max}^p$ and $\sigma_{min}^p$ are the maximum and minimum principal stress, respectively. If the stress state at a point in the tissue is perfectly bi-axial then $\sigma_{max}^p = \sigma_{min}^p$ so that $\tilde{\sigma} = 0$. On the other hand, if $\tilde{\sigma} = 1$ the stress state is perfectly uniaxial, with $\sigma_{min}^p = 0$.

### 4.12 Statistical analysis and data representation

Statistical significance was computed using the Mann-Whitney $U$ test. All error bars represent the standard deviation of three or more biological replicates. Asterisks (*) indicate statistical significance between conditions of $p<0.05$. All data analyses, including graphical representations, were performed using Excel (Microsoft, Redmond, WA); statistical analysis was performed using custom macros written in R programming language (R Development).
5 Results

5.1 In vivo-like morphology of heart cells recapitulated in vitro using nanoengineered aligned collagen inhibits hyperproliferation

The ECM plays a critical role in cell niches during development, homeostasis, disease, and repair. One mode in which the ECM communicates with cells is through contact guidance. Topographical cues facilitate integrin binding of cell focal adhesions to proteins on the ECM which ultimately affect cell morphology. In order to investigate the impact of contact guidance-mediated cell alignment, we cultured heart cells on collagen-deposited borosilicate glass substrates in both unaligned and aligned pseudo-three-dimensional geometries (Supplementary Figure 1). Unlike conventional techniques for depositing ECM onto substrates for monolayer cell culture, including ECM adsorption onto surfaces via coating and contact printing, these collagen machine-deposited matrices maintain mechanical strength over time and do not rapidly degrade. Furthermore, the matrices are precisely oriented in a layered and striated pattern at the nanometer scale, creating an architecture which closely mimics the native ECM of the heart.

Rationalizing that the ECM is critical in providing anchorage to focal adhesions in cardiomyocytes and non-myocytes to allow for their highly dense, mechanically intensive, and aligned architecture, we tested whether the architecture of the ECM played an additional role of controlling the population dynamics of heart cells. Two cell types, rat neonatal (rN) heart cells, and human Embryonic Stem Cell (hESC)-derived heart cells were seeded and cultured on the collagen matrices for 7 days (Figure 4 A-G). Bare glass and unaligned collagen substrates were used as controls. For both rN and hESC-derived heart cells, we found non-cardiomyocytes (non-CM) cultured on glass had the greatest degree of total cell density (3-fold greater than aligned collagen, and 2-fold greater than unaligned collagen). Non-CM cultured on unaligned collagen had 1.5-fold greater total cell density compared to aligned collagen. There were no significant differences, however, in total CM density over the three substrates (Figure 4 H, I).

To rule out selective apoptosis, we measured the percentage of cell proliferation directly by staining for proliferation marker Ki67 in the hESC-derived heart cells. As expected, non-CM
expressed greater Ki67+ expression (2-fold) on unaligned collagen compared to aligned collagen. Interestingly, CM also expressed greater Ki67+ expression (2-fold) on unaligned collagen (Figure 4 J). This suggests that along with the ability of aligned ECM to inhibit hyperproliferation of non-CM, it may also have the ability to prevent the proliferation of CM, and in doing so, help drive maturation to an adult-like phenotype. These results indicate that the topographical cues provided by the ECM has a critical role to play in maintaining the cardiomyocyte population within the heart and to prevent overgrowth of non-CM cell types as is commonly observed in culture of primary heart cells and pluripotent stem cell (PSC)-derived heart cells. Typically, this population consists of smooth muscle cells, endothelial cells, and cardiac fibroblasts; hyperproliferation of some of these cell types, along with fibroblast-derived myofibroblasts, are often associated with heart disease.

Many studies have described the ability of topographical cues to direct the elongation and orientation of cells (D.-H. Kim et al., 2010; Pot et al., 2010). In order to quantify cell elongation in CM, we calculated for each cell the ratio of major axis to minor axis length and categorized it into one of three bins. As expected, the trend indicates that for both rN-CM and hESC-CM, aligned collagen facilitates cell elongation better than unaligned collagen (Figure 4 K). We also investigated the orientation of CM with respect to the direction of collagen alignment. Once again as expected, for both rn-CM and hESC-CM, cell orientation was highly observed in aligned collagen compared to unaligned collagen (Figure 4 L, M).

Effects of cardiac cell alignment on the direction of AP propagation have been previously reported (D.-H. Kim et al., 2010). As a final study we confirmed these findings with our striated and layered nanoscale collagen matrix. After culturing hESC-CM for 7 days, we used optical mapping techniques to observe AP propagation. As can be observed in Supplementary Figure 2, the CM cultured on the unaligned collagen had an AP propagation that was out of phase with neighbouring CM patches. The CM on aligned collagen on the other hand, had AP propagation parallel to the direction of collagen alignment within and between CM patches. Collectively these studies support our interpretation that the ECM is critical in controlling aspects of cell proliferation kinetics, morphology, and electrophysiological function in the heart cell niche.
Figure 4. In-vivo-like aligned morphology of cardiomyocytes and non-cardiomyocytes can be recapitulated in vitro using aligned ECM-patterned substrates. [a-c] Topographical cues of patterned collagen in 2-D facilitate contact-guidance-mediated cell elongation and alignment. On glass, unaligned collagen, and aligned collagen substrates, rat neonatal heart cells show significantly diverse levels of density due to proliferation over 7 days. Bi-directional white arrow set indicate orientation of collagen alignment on substrates. Immunofluorescence shows Phalloidin-stained actin filaments (red), Cardiac TroponinT (green) and DAPI-stained nuclei (blue). [d] Cell cytoskeleton alignment and striations (small white arrowheads) are observed in elongated nr-CM on aligned collagen. [e] AFM micrograph of aligned collagen patterned surface on glass slide. [f, g] Heart cells derived from hESC respond to aligned and unaligned collagen. Immunofluorescence shows proliferation marker Ki67 (red), cTnT (green) and nuclei (blue). [h, i] Quantification of rn and hESC-derived heart cell growth on both aligned and unaligned substrates. [j] % Ki67+ heart cells on both aligned and unaligned substrates. [k] Cell elongation of rn-CM and hESC-CM. Measure of cell elongation is the ratio of the major axis to the minor axis of a cell. [l, m] Cell orientation of rn-CM and hESC-CM on both aligned and unaligned substrates. Measure of cell orientation is relative to alignment of patterned collagen.
5.2 Rational design of high content screening platform, capable of generating, cultivating, and assaying self-assembling microtissues of specialized 3-D geometries

Studies have repeatedly shown the impact of ECM three-dimensionality on the phenotype, genotype, and also the functionality of contained cells, independent of cues from soluble factors. We reasoned that to engineer an accurate heart cell niche we would need to transition our findings from pseudo-3-D substrates to truly 3-D cell-encapsulating ECM geometries. In order to do this efficiently, we exploited the cells’ ability to remodel pliant ECM during phases of growth and proliferation. Through gel compaction caused by cell traction forces (Supplementary Figure 3), dissociated heart cells encapsulated in a gel will go through several phases: 1) recovery of actin filaments and extension of filopodia, 2) accumulation and assembly of cell adhesion molecules, gap junctional and contractile proteins, and finally 3) excitation-contraction coupling which permits the cardiac tissue to propagate action potentials and contract in unison (Radisic et al., 2004). We created an array of isolated microwells of various sizes, containing specific node geometries, to restrain the remodelling of localized pockets of cell-laden collagen within the microwell recessions (Figure 5). We adapted this system to generate miniaturized microtissues of various geometries in a higher-throughput manner (Supplementary Figure 4, and Figure 6). Using a combination of this microfabricated platform and computational modelling, we aimed to design and generate a miniaturized 3-D system which allowed cells to maintain high sarcomere expression, aligned microtissue architecture, and integrated electromechanical stimuli in a relatively simple form factor.

As a preliminary step, we explored in silico strategies to predict and evaluate spatially patterned mechanical stresses distributed within various 3-D tissue geometries. We chose two simple microtissue geometries to model using a finite element model-based simulation of microtissue contractility (Figure 6) (Deshpande, McMeeking, & Evans, 2006): microtissue geometries under 1) bi-axial and 2) uni-axial intratissue tension forces (BITF and UNITF respectively). An important insight the model provides is that mechanical stress produced by intratissue tension via cell traction forces is a strong modulator of the cytoskeletal and ECM protein structure within a tissue (Legant et al., 2009). For our system, the model determined areas of stress in both the BITF and UNITF microtissues as well as areas of aligned tissue and sarcomere expression. Our
model simulation determined that bordering regions of our BITF microtissue geometries experienced the highest stress relative to the centre (Figure 7). The lowest stress was predicted to be in the centre of the BITF microtissues, with a graded continuum of mechanical stress between these points of minima and maxima force of tension. In the UNITF microtissue, high stress was exhibited throughout the length of its geometry to produce a uniformly high regime of intratissue tension. The model further predicted that for both microtissue geometries under bi-axial and uni-axial intratissue tension forces, cardiac sarcomere protein expression is patterned in areas of high mechanical stress (Figure 7).

In order to experimentally verify these predictions, we generated microtissues in microfabricated arrays of wells within a PDMS mould (Figure 5). We designed disc inserts containing the recessed arrayed microwells and placed them inside of a universal 24-well TCTP multiwall plate. NR heart cells were then suspended in a fully-defined non-polymerized collagen matrix, applied over the microfabricated substrate, and centrifuged to force the cells into the recessed microwells (Figure 5). The excess collagen was removed and the remaining pockets of cell-laden collagen were polymerized. We observed cells begin to extend filopodia and remodel the surrounding collagen matrix, and within 3 days the microtissues had formed and hit a plateau in morphology (Supplementary Figure 5). As expected, immunostaining for cardiac sarcomeric proteins alpha-actinin and cTnT revealed high expression in areas of high stress as predicted by our model (Figure 8). Additionally, in areas where the model predicted tension-induced alignment due to high uniaxial stress, we observed elongated and oriented cell alignment parallel to modelled localized tension force lines.
Figure 5. Platform microfabrication method and process flow for microtissue seeding and generation. A PDMS substrate is replicated from the original master and then modified with tapered heads. A negative polyurethane-based master is generated and used as the final master mould. Substrates are then prepared in 24-well plates and sterilized. Cell laden collagen is centrifuged into recessions of microwells and allowed to remodel and grow.
Figure 6. Microtissue formation in microfabricated platforms. [a] Cells are seeded and centrifuged into recessions and allowed to remodel to form microtissues based on node geometries. [b] Platforms with deflecting posts can be used to measure forces exerted by tissue and to also constrain tissue remodelling at various degrees. [c] Microtissues can be arrayed on a common surface to increase samples per well. [d, e] Microtissues can be fixed, permeabilized, stained, and imaged within the microtissue platform. Immunofluorescence shows Phalloidin-stained actin filaments (red), Cardiac TroponinT (green) and DAPI-stained nuclei (blue).
Figure 7. Simulations of candidate microtissue designs. [a] Microtissue platform design to permit bi-axial intratissue tension forces. Finite element simulations of [b] the bi-axial stress state (represented by a non-dimensional effective stress $\tilde{S}$) and [c] the distribution and alignment of sarcomeres in the bi-axial microtissue (represented by the output parameter $\Pi$) defined as the difference between maximum and mean sarcomere activation level. [d] Microtissue platform design to permit uni-axial intratissue tension forces. Finite element simulations of [e-f] the uni-axial stress state and [c] the distribution and alignment of sarcomeres in the uni-axial microtissue.
Figure 8. Predicted stress gradients within microtissues reflect patterned intratissue cardiac sarcomeric protein levels. Microfabricated substrates can be used to generate fully-defined self-assembling microtissues of specialized 3-D geometries. [a] Finite element modeling of the bi-axial and uni-axial intratissue tension force microtissues (BITF µTissue and UNITF µTissue respectively) indicating predicted intratissue stress and alignment gradients. [b] Process flow diagram outlining microtissue generation, cultivation, and assay. [c-e] Tension forces in 3-D microenvironments facilitate remodeling and alignment of heart cells. BITF µTissue [c, d] expressing Alpha-actinin (green), and DAPI-stained nuclei (blue) and UNITF µTissue [e] expressing cTnT (green), and DAPI-stained nuclei (blue).
5.3 Cardiac MicroWires (CMW) can be engineered to mimic healthy and disease-like human electrophysiological function

Based on our simulations and empirical validation, the UNITF microtissues exhibited high cytoskeletal organization measured via cardiac sarcomere expression and tissue alignment. As a next step, we carried forward the UNITF microtissue design in our subsequent studies after some minor design modifications to allow for a longer and robust “wire-like” structure we term Cardiac MicroWires (CMW). The two posts within the microwells, which restrict the remodelling process and provide mechanical resistance during synchronous contraction, were integrated with platinum wire electrodes to provide electrical point stimulation capability. The entire system was outfitted within a 24-well tissue culture plate for ease of use (Figure 9 A, B).

Following initial seeding of cell-laden collagen, CMW remodelled within three days (Figure 9 C). Along with time in culture (Supplementary Figure 5), we found that initial collagen concentration had an effect on the remodelling dynamics of CMW (data not shown). Higher concentrations of collagen prolonged the time and extent of remodelling. Overall sarcomere expression in the CMW microtissue was observed to be high and spatially homogeneous in comparison to the BITF microtissue as expected. Quantified expression of cTnT was 2.5-fold higher in CMW compared to the control BITF (Figure 9 E). Cell elongation was also significantly higher in the CMW system, likely due to the tension forces acting on the cells additional to the aligned collagen architecture (Figure 9 F). As expected, cells in CMW demonstrated visibly higher alignment compared to cells in BITF microtissue (Supplementary Figure 6, Figure 9 G).

We next generated CMW composed of hESC-derived heart cells and observed similar remodelling characteristics. To ensure that the cells were aligning the collagen-based ECM after three days of remodelling, we assessed the CMW using the LC-PolScope quantitative birefringence imaging system. In our PolScope images (Figure 10), the colour of the pixel determines the orientation angle of the fibrillar collagen. We noted that the fibrillar collagen within the CMW was indeed aligned in parallel to the length of the CMW as expected.

Excitation Threshold (ET) and Maximum Capture Rate (MCR) were shown to improve significantly when hESC-derived heart cells were dissociated form their original aggregates and
cultured as CMW (Figure 11 A, B). MCR improved even further when the CMW were electrically point stimulated with a biphasic square wave pulse for three days. CMW had an intrinsic spontaneous beating frequency of ~1 Hz which is an expected baseline for human CM (Figure 11 C). Using drugs of known effects we perturbed CMW and optically mapped their response with a voltage-sensitive dye. Addition of Norepinephrine to CMW doubled the rate of activation rate, while Lidocaine reduced and nearly abolished the activation of the CMW (Figure 11 D). We recorded conduction velocities of CMW and compared them to healthy and diseased conduction velocities of the human heart (Table 1). Remarkably, CMW conduction velocity (47.4 ± 12.4 cm/s) was found to be comparable to that of a healthy human heart (46.4 ± 2.7 cm/s).

As an extension, we studied and manipulated the dynamics of AP propagation in CMW. Normal AP propagation in CMW initiates in the loop of one end, converges, traverses down the length of the wire, and then diverges at the neck of the opposite loop (Figure 12 A). In some cases, we were able to observe the AP propagation dynamics perturbed by physical deformities similar to reentrant waves in arrhythmias caused by scar formation. This conduction block was observed at the neck of the loop as can be seen in Figure 12 B. We also determined that AP propagation direction in CMW could be manipulated using electrical point stimulation. Starting with CMW with spontaneous AP traversing from left to right, we reversed the AP direction by electrically pacing from the right side (Figure 12 C, D).
Figure 9. Rational design of microfabricated platform for generating, cultivating, and assaying CMW in a microenvironment that maintains cTnT expression and tension force-mediated cell alignment. [a, b] A 24-well bioreactor system composed of platinum wire electrodes embedded in a microfabricated substrate is used to contain CMW from the point of seeding to cultivation to assaying. [c] CMW are generated and cultivated for 7 days in culture; the remodeling phase is completed within 3 days. [d] CMW under uni-axial tension remodel reproducibly in culture and express cardiac-specific proteins. Tissue architecture reveals tightly packed cellular alignment and cTnT expression (green) in CMW generated using rn-CM. [e] CMW express cTnT about 2-fold greater than BITF microtissue. [f, g] Cardiomyocytes in CMW under uni-axial tension exhibit an increased cell elongation and cell alignment compared to the control (BITF microtissues under bi-axial tension).
Figure 10. Fibrillar collagen content of CMW using the LC-PolScope quantitative birefringence imaging system. [a-h] In PolScope images, pixel colour is related to angle of birefringent fibrillar collagen in the CMW. Both bright field and LC-PolScope images are shown with colour wheel indicating angle of fibrillar collagen. [a-d] CMW held taut shows unidirectionally aligned collagen. [e-h] CMW which has compacted maintains fibrillar collagen alignment.
Figure 11. Electrical point-stimulation of CMW composed of hESC-CM result in improved function; CMW exhibit electrophysiological response similar to that of in vivo tissue. [a, b] CMW composed of hESC-CM; along with morphology, cTnT expression is similar to that of CMW composed of rat-CM. Excitation threshold and maximum capture rate of non-dissociated hESC-CM aggregates, non-stimulated CMW, and stimulated CMW. [c, d] Spontaneous activation rate of hESC-CM CMW revealed a rate of approximately 1 Hz. CMW were also responsive to high frequency pacing and drugs with known effects. Optical mapping was employed to determine activation rate of hESC-CM CMW. Measurements revealed a spontaneous activation rate of 1 Hz as expected for human cardiomyocytes. CMW were also responsive to high frequency pacing and respond as expected to drugs with known effects. Norepinephrine increases the rate of activity and Lidocaine decreases the rate relative to the baseline control. Location trace of recording and timescales are indicated.
<table>
<thead>
<tr>
<th>Subject</th>
<th>Conduction Velocity (cm/s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiomyopathic human heart during ventricular fibrillation</td>
<td>25 ± 4.0</td>
<td>(Kumaraswamy Nanthakumar et al., 2007)</td>
</tr>
<tr>
<td>Cardiomyopathic human heart during pacing</td>
<td>41 (min) – 87 (max)</td>
<td>(Kumaraswamy Nanthakumar et al., 2007)</td>
</tr>
<tr>
<td>Healthy human heart</td>
<td>46.4 ± 2.7</td>
<td>(Durrer et al., 1970)</td>
</tr>
<tr>
<td>Human CMW</td>
<td>47.4 ± 12.4</td>
<td>-</td>
</tr>
<tr>
<td>Healthy human heart - Purkinje fibres</td>
<td>≈ 2000</td>
<td>(Durrer et al., 1970)</td>
</tr>
</tbody>
</table>

Table 1. Comparison of conduction velocities in vivo and the CMW system. Values and ranges of normal and pathophysiological conduction velocities were obtained from the literature. Values for CMW were measured from three experiments via optical mapping. Microengineered CMW were found to exhibit conduction velocities on par with that of a healthy heart.
Figure 12. Action potential propagation dynamics of CMW can be modulated using conduction-blocking obstructions, and propagation direction can be reversed via electrical point-stimulation. [a] Propagation of normal CMW. (iii) for each panel depicts a time lapse of the AP propagation. [b] AP propagation of CMW observed to be obstructed by a conduction block resulting in a re-entrant wave-like system. [c, d] Direction of spontaneous AP propagation of [c] normal CMW can be reversed using [d] electrical point stimulation.
5.4 Composition of CMW input cell population determines tissue morphology and gene expression through cell-cell and cell-ECM interactions (NKX2-5+ and CD90+ hESC-CM mixing studies)

As a final study, we decided to sort our hESC-derived heart cells in order to generate tissues with specific input populations consisting of CM and cardiac fibroblasts (cFB). We applied our cardiac differentiation protocol to an NKX2-5-GFP reporter hESC line that contains the EGFP cDNA inserted into the NKX2-5-GFP locus of HES3 hESC (Dubois et al., 2011; Elliott et al., 2011). At the end of our differentiation protocol on day 20, we dissociated the aggregates and sorted them using Flow Activated Cell Sorting (FACS). We generated CMW of specific CM to cFB ratios and control aggregates of tissue (without exogenously supplemented ECM) at specific ratios of CM to cFB (see Table 2). A control set of non-dissociated hESC-CM aggregates were also maintained. CMW were generated as previously described, and microtissue aggregates were generated in the AggreWell system. A suspension of single cells were centrifuged into the AggreWells at a cell density (of 0.6 x 10^6 cells/mL) which yielded aggregates of 500 cells per microwell.

Both the CMW and aggregate microtissues were cultured over seven days. We observed familiar remodelling kinetics compared to our previous experiments, however, the tissue morphology showed differences between the tissue composition conditions. As the composition of CD90+ (cFB) increased, we observed a tighter, integrated tissue morphology under higher tension. Many of the CMW consisting of 75% CD90+ cells snapped from failure due to the high tension forces exerted by the cFB. Additionally, the majority of 75% CD90+ CMW did not display synchronous contractions, and those that did exhibited very low spontaneous activation rates (less than 1 Hz). In the condition with 100% NKX2-5+ cells, CMW formed unstable tissue with low cell-cell integration asynchronous contractions (Figure 13 A-C). Globular contracting aggregates, likely clonal populations of proliferating CM, separated by patches of collagen were observed throughout the CMW. As the percentage of CD90+ cells decreased to 25%, however, the CMW took on a more robust architecture with synchronous contractions resembling in vivo-like tissue morphology (Figure 13 D, E).
In order to determine further effects of CMW formulation on these cells, we examined gene regulation of key cardiac markers. We initially looked at control cardiomyocyte markers for determining dilution consistency of input cell composition. Conditions ‘A’, ‘B’, ‘C’, ‘D’ correspond respectively to 100, 75, 50, and 25 percent NKX2-5-GFP+ cells with the remainder consisting of CD90+ cells as per Table 2. As expected CM marker expression in CMW decreased with the decrease of initial seeded CM composition (Figure 14). Additionally, the ratios of CM seem to be generally maintained in the CMW as indicated by NKX2-5, cTnT, SIRPA, and IRX4. With respect to our control, condition ‘agg’, which was a dissociated and sorted aggregate composed of 25% CD90+ cells, our corresponding CMW ‘B’ consisting of the same ratio was comparable in control CM gene expression, save for HCN4 which is expressed primarily in the pacemaker region of the heart.

We then looked at the effects of maturation via a panel of genes including markers indicative of healthy CM maturation. When comparing condition ‘B’ to control ‘agg’, we see a decrease in α-MHC (MYH6) and an increase in β-MHC (MYH7), which may indicate CM maturation. Supporting this is the increase in genes MYL2, KIR2-1 and SCN5A. Another hallmark of CM maturation is the decrease in ISL1 expression which occurs when CM transition from an embryonic to adult-like phenotype; however, this was not observed in our 7 day time-point. Additionally, we do not see the up-regulation of genes SERCA2 and RYR2 which are key regulators of calcium regulators in the sarcoplasmic reticulum. It is important to note that this final mixing study is still preliminary (n=1). Replicate experiments are currently underway with an additional 14-day time-point.
<table>
<thead>
<tr>
<th>Conditions</th>
<th>%NKX2.5+</th>
<th>%CD90+</th>
<th>Tissue composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CMW</td>
<td>Sorted</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>CMW</td>
<td>Sorted</td>
<td>75</td>
</tr>
<tr>
<td>C</td>
<td>CMW</td>
<td>Sorted</td>
<td>50</td>
</tr>
<tr>
<td>D</td>
<td>CMW</td>
<td>Sorted</td>
<td>25</td>
</tr>
<tr>
<td>US agg</td>
<td>Aggregated in AggreWell</td>
<td>Sorted</td>
<td>75</td>
</tr>
<tr>
<td>US</td>
<td>Aggregated in AggreWell</td>
<td>Non-sorted</td>
<td>-</td>
</tr>
<tr>
<td>Non-dissociated aggregates in low cluster 6-well plate</td>
<td>Non-sorted</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. CMW mixing studies to determine effects of cell-cell and cell-ECM interactions. NKX2-5-GFP+ cells and CD90+ cells were sorted and mixed at specific ratios in CMW and aggregates. Heart cells from cardiac differentiation of hESC were sorted for cardiac myocytes and cardiac fibroblasts and used to generate either CMW (A, B, C, D), or aggregates (agg). Non-sorted controls were also used (US agg, US).
Figure 13. CMW composed of various input cell compositions result in distinct morphologies. [a-c] CMW composed of pure NKX2-5-GFP+ cells present a globular morphology with non-integrating colonies of CM. [d, e] CMW composed of 75% NKX2-5-GFP+ cells and 25% CD90+ cells produced well-integrated tissue with robust architecture.

Figure 14. Gene expression of control cardiomyocyte markers for determining dilution consistency of input cell composition. A, B, C, D correspond respectively to 100, 75, 50, and 25 percent NKX2-5-GFP+ cells with the remainder consisting of CD90+ cells. As expected CM marker expression decreases with the decrease of CM composition.
Figure 15. Gene expressions of heart cell markers for determining maturation of input cell composition. A, B, C, D correspond respectively to 100, 75, 50, and 25 percent NKX2-5-GFP+ cells with the remainder consisting of CD90+ cells. The upregulation of certain markers in our ‘B’ CMW relative to the ‘agg’ control with comparable input cell composition is indicative of tissue maturation.
6 Discussion

6.1 Discussion

As in the hematopoietic system, emerging data suggests that robustness and responsiveness in the cardiovascular system is a property of the system as a whole and not of individual cells (Banerjee, Yekkala, Borg, & Baudino, 2006; T. A. Baudino et al., 2008; Kamkin, Kiseleva, Lozinsky, & Scholz, 2005; Kohl, Camelliti, Burton, & Smith, 2005; Matsusaka, Katori, Inagami, Fogo, & Ichikawa, 1999; Ottaviano & Yee, 2011; Van Wamel, Ruwhof, Van Der Valk-Kokshoorn, Schrier, & Van Der Laarse, 2000). The developing and adult heart each contains dynamic cell niches that are suited primarily for growth and differentiation or maintained function, respectively. The developing heart is able to allow gradients of small molecules to specify differentiation along various cardiovascular lineages while the adult heart is, to a limited extent, capable of dynamically maintaining a balanced composition of cardiomyocytes, cardiac fibroblasts, smooth muscle cells, and endothelial cells within a highly ordered extracellular matrix (ECM) architecture (Banerjee, Fuseler, Price, Borg, & Baudino, 2007; T. a Baudino, Carver, Giles, & Borg, 2006; Leri, Kajstura, & Anversa, 2005; Tsuruda, Imamura, Hatakeyama, Asada, & Kitamura, 2010). It is through the result of many complex cell-cell and cell-ECM interactions that the heart is able to maintain homeostasis and, to a limited extent, repair in response to ischemic injury.

Conventional in vitro model platforms for drug screening and toxicity testing typically use tissue culture treated polystyrene surfaces coated with a basal membrane (Walsh, Rich, & Coffman, 2009). These two-dimensional substrates lack topographical cues and often have elastic moduli orders of magnitude greater than the native substrate of the targeted cell type (Discher, Janmey, & Wang, 2005; Fu et al., 2010; Raab, Shin, & Discher, 2010). Additionally, targeted cell types in these assays are cultured either on their own, with conditioned media from stromal cells, or with a physiologically inaccurate proportion of supporting cell types usually found in the native niche of the target cell. Many of the paracrine factors and associated signalling networks which enable normal characteristics of the target cell are missing, and thus prevent a reliably accurate response to external perturbation, rendering potential hits in a screen seemingly ineffective, or revealing
candidates which turn out falsely positive. Lastly, and especially in the case of the heart cell niche, gradients of electrical (Panáková, Werdich, & Macrae, 2010) and dynamic mechanical forces (Kurazumi et al., 2011; Salameh & Dhein, 2012) provide critical electro- and mechano-transduction signalling during development through to maturation, followed by disease and repair.

Studies have shown the effects of topographical cues and contact guidance of three dimensional ECM on various cell types (Biela, Su, Spatz, & Kemkemer, 2009; Matthew J Dalby, Riehle, Sutherland, Agheli, & Curtis, 2003; Grinnell, Nakagawa, & Ho, 1989; D.-H. Kim et al., 2010; Pot et al., 2010). Along with cell morphology (Del Álamo, Norwich, Li, Lasheras, & Chien, 2008; McNamara et al., 2012), rate of proliferation (Peyton, Ghajar, Khatiwala, & Putnam, 2007), migratory ability (S. Li, Huang, & Hsu, 2005), differentiation potential (Khatiwala, Kim, Peyton, & Putnam, 2009; Lam et al., 2010), drug responsiveness (Ohbayashi et al., 2008), and juxtracrine signalling (Mancini & Di Battista, 2006) have all been shown to be affected by ECM-mediated mechanotransduction. Additionally, the importance of paracrine signalling provided by supporting cells in specialized niches have been reported numerous (Accornero et al., 2011; Jackson, Schiesser, Stanley, & Elefanty, 2010), including important factors provided by cardiac fibroblasts (Noseda & Schneider, 2009) and Mesenchymal Stromal Cells (Sassoli et al., 2011) for CM survival and proliferation. To determine true efficacy of small molecule-based therapeutics, the niche of target cell populations must be strictly recapitulated and controlled.

We outline a microengineered tissue-mimetic platform which integrates the basic components of the heart cell niche and allows for medium-throughput high-content screening of cells and small molecules.

Design strategies for generating effective in vitro models have lacked in at least one of three fundamental criteria which include: a) recapitulation of an in vivo-like microenvironment specifically engineered for the input cell population, b) medium- to high -throughput assay, and c) high content nature of output parameters. Our rationally designed system fulfils each of these criterions and has been experimentally validated through phenotypic and functional characterization.

We first confirmed the role of ECM topography as important in maintaining a homeostatic composition of myocytes and non-myocytes along with promoting aligned cell morphology and
function. Cell-ECM and cell-cell interactions in vivo are especially important in the heart cell niche due to the organ’s reliance on structure for function, and a balanced ratio of the many cell types found in the heart (Banerjee et al., 2007; T. a Baudino et al., 2006; Leri et al., 2005). Recapitulating ECM architecture that is physiologically accurate is critical in in vitro models because there are proteins on the ECM that facilitate integrin binding critical to the survival, function, and growth of cells (E. C. Goldsmith et al., 2003; S.-H. Kim, Turnbull, & Guimond, 2011; Laser et al., 2000). During development, maintaining ratios of CM and non-CM through proliferation rate is carried out relatively easily, however, in times of injury caused by MI, fibroblasts over-proliferate in ischemic regions of high stiffness in order to maintain local mechanical integrity (Hadjipanayi, Mudera, & Brown, 2009). In our initial study, we observed the lowest rate of proliferation in the non-CM population when cultured on aligned collagen that mimicked the organized structure of healthy myocardium. We have established in these preliminary 2D studies that the alignment of heart cells is beneficial to maintaining an appropriate population dynamic in culture, specifically through the inhibition of fibroblast proliferation.

In order to identify appropriate microtissue geometries to recapitulate aligned cardiac tissue as observed in our 2D studies, we employed a computational simulation of microtissue contractility to predict and evaluate areas of high stress and cardiac protein expression. Through the model, we linked areas of high stress with high cardiac protein expression and cell alignment and then experimentally confirmed predictions of increased cardiac sarcomere expression, via alpha-actinin and cTnT expression, in areas of increased uni-axial stress within our microtissues. We used a microfabrication approach to generate microscale wells to contain dissociated cells encapsulated in a collagen matrix. The suspended single cells recovered surface ion channels and receptors, along with the synthesis and assembly of lost conductive and contractile apparatus over time and remodelled into a contracting tissue. Selecting the microtissue configuration consisting primarily of uni-axial intra-tissue tension forces, we adapted the CMW design to a multiwell plate. Miniaturization of our platform eliminates the need for vascularization as microtissue diameter is below the diffusion limitation threshold of 150 um. The lack of a diffusion barrier also allows us to exploit traditional immunofluorescence and imaging techniques in situ. Additionally, the input cell population needed for assaying is minimal compared to conventional three dimensional in vitro models. Lastly, we integrated
electromechanical stimuli in our system via pacing point stimulation electrodes and passive stretching. The integration of key components of the cardiac niche has produced a physiologically relevant system for high content screening.

We functionally assessed the CMW and determined normal spontaneous response and response to drugs. Additionally, CMW cultured for 7 days exhibited a conduction velocity of $47.4 \pm 12.4$ cm/s, on par with healthy human heart tissue. Manipulation of AP propagation dynamics was also achieved and leaves available the possibility of engineering disease like physiologies using strategic geometries and insults to the microtissue. Specifically, areas of conduction block can be created on the tissue to mimic MI, or re-entrant waves can be geometrically designed to observe effects on AP propagation dynamics.

We finally made preliminary observations on the effects of input cell population on the formation of CMW. Pure CM were not effective in generating synchronously contracting aligned and integrated CMW. CMW composed of 100% NKX2-5-GFP+ cells visibly exhibited weak contractile forces due the asynchronous contraction of segregated clusters of CM, and because of the numerous patches of empty collagen not containing cells, they easily failed over time due to tension. As cFB were introduced into the microtissue formulation, however, the CMW took on a more normal morphology with synchronicity and the formulating cells were integrated well. However, when the percentage of cFB was too high (75%) CMW did not contract synchronously at a regular 1 Hz. Although this formulation generated well integrated tissue, CMW snapped due to the high tension forces exerted by the high number of cFB. This high level of tension normally induces cFB to secrete ECM to reinforce the biomechanics of the local area; however, our system may not be giving the cFB enough time to do so. This may be another potential method of generating disease-state CMW for MI models. We examined a panel of maturation markers and the initial results suggest a maturation effect applied by the CMW. We will need to extend the time of study to perhaps 14 days to fully observe the effects manifested in more than just a few markers of maturation, these studies are on-going.
6.2 Future studies

We would like to integrate force of contractility and real-time electrophysiological readouts into the CMW system to allow for a true high-content gold-standard in vitro heart model. Specifically, for the next iteration of the design a rigorous comparison with in vivo parameters will need to be made, including AP signature, fractional shortening, substrate stiffness, paracrine and autocrine signaling profiles, tissue composition, and gene/protein expression. This will need to be quantified as well, and comparisons made to in vivo data where possible. We have made some progress with real-time force measurements using deflecting posts (Figure 16) (Boudou et al., 2011). We are also looking into identifying candidate small molecules (for regeneration, maturation, and function) through literature curation and preliminary screening techniques. Medium-throughput screens will be performed using the CMW system and follow-up studies will be conducted on hits using a CMW-based MI model. Finally, we would like to push the system to determine intratissue cell-cell interactions between CM, cFB, EC, and SMC via secreted factor profiling.
Figure 16. Temporal evolution of CMTs constructed in collagen gels and tethered to rigid (k = 0.45 mN/mm) cantilevers. (A) Representative images depicting the time course of a contracting CMT. (B) Representative recording of the tension as a function of the time of a CMT on day 5. (C) Temporal evolution of the spontaneous beating frequency and (D) of the contraction duration of beating (contraction and relaxation) (Boudou et al., 2011).
7 Conclusions

Through understanding the critical components of the myocardial niche, we have produced a rational design of a microtissue in vitro model that is capable of very closely mimicking healthy human heart tissue. The microscale CMW system draws on the importance of an aligned three-dimensional ECM which is able to support interacting cardiomyocytes and non-myocytes under dynamic electromechanical forces. This system uses minimal amounts of input material and provides high-content output parameters including cell morphology, cardiac marker expression via in situ immunofluorescence, electrophysiological readouts, and gene expression. A physiologically accurate in vitro model such the CMW system will facilitate effective screening studies for small molecule-based therapeutics with precise responsiveness.


9  Appendices

**Supplementary Figure 1.** AFM micrographs of the 4 candidate topographies of collagen deposited glass slides.
Supplementary Figure 2. Optical mapping of aligned and unaligned hESC-CM. (i) Phase contrast and (ii) isochronal map of [a] unaligned and [b] aligned hESC-CM during spontaneous electrical activity. [c] Larger aggregates also formed on the aligned slides but did not grow and extend outwards.
Supplementary Figure 3. Gel compaction occurs as a result of collective tensile forces exerted through the focal adhesion points of cells contained in the collagen gel. As the cells grow, the surrounding tensile forces increase [a], and as a result the entire gel remodels [b, c].
Supplementary Figure 4. Microtissue well dimensions. Designs for the Bi-axial microtissue tension forces (BITF), Uni-axial microtissue tension forces (UNITF), and Cardiac MicroWire (CMW).
**Supplementary Figure 5. Cardiac MicroWire (CMW) width over time.** CMW remodel over three days and tissue width plateaus at approximately 150-200 µm.

**Supplementary Figure 6. Alignment of microtissues under bi-axial and uni-axial tension forces.** Cells in control microtissues (BITF) show no nuclei elongation and alignment, whereas cells in aligned CMW exhibit high alignment and elongation. DAPI-stained nuclei are shown in blue.