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Microfluidic Cardiac Circulation Model (μCCM) for Functional Cardiomyocyte Studies

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Abstract— **Physiological heart development and cardiac function rely on the response of cardiomyocytes to mechanical stress signals during hemodynamic loading and unloading. These stresses manifest themselves via changes in cell structure, contractile function and gene expression. Disruption of this well balanced stress-sensing machinery due to various pathological conditions results in contractile dysfunction, cardiac remodeling and heart failure. In order to study signaling mechanisms involved in the pathogenesis of various manifestations of Cardiovascular Disease (CVD), there is a need for physiologically relevant *in-vitro* models. To accomplish this goal, we have developed a Microfluidic Cardiac Circulation Model (μCCM) that integrates mechanically loaded cardiomyocytes with fluid flow and a circulation network.**

I. Introduction

Cardiomyocytes have the ability to sense mechanical loads during normal hemodynamic loading and unloading and convert it to intracellular signals that control cell phenotype and muscle mass through hypertrophy. Pathological conditions like myocardial infarction and ischemic injury result in disruption of cardiomyocyte mechanotransduction cascade, which in turn results in cell loss, tissue remodeling and deterioration of cardiac function[1]. Despite significant progress in understanding cardiomyocyte signal transduction, several important questions for example: Does the change in mode of loading (from pulsatile to continuous) adversely affect cardiomyocyte function? Why does hypothermic reperfusion induce cardioprotection following ischemic injury? have not been fully understood. The tradeoff between specificity and biological relevance necessitates investigation at different levels of cellular organization. Cell-based studies are essential to provide highly specific information with regards to molecular mediators and targets for drug discovery. Current *in-vitro* cell culture systems enable generation of such information but fail to adequately recreate the complex *in-vivo* cardiomyocyte environment by replicating the effects of mechanical loading in the intact heart.

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The current state of cardiomyocyte *in-vitro* models is predominantly based on glass slides or tissue culture dishes under static conditions[2]. Most studies utilize neonatal cardiomyocytes maintained in two-dimensional culture forming randomly oriented cell - extra-cellular matrix (ECM) attachments. Accurate replication of the *in-vivo* environment requires consideration of the following factors: Tissue orientation and direction of stretch are critical determinants of cardiomyocyte signaling, therefore randomly oriented cultures cannot replicate physiological loading. Fluid flow and shear play important roles in determining cardiomyocyte cell structure, function and phenotype, hence perfused systems may be more representative of the *in-vivo* environment. In addition to these factors, the model system requires a certain degree of flexibility to enable variations in mechanical loading and fluid flow to replicate various conditions including hypoxia, ischemia and changes in hemodynamic loading and unloading.

Some of the aforementioned issues have been addressed; albeit individually. Patterning of ECM proteins using techniques like microcontact printing and lithography to define areas of cell attachment and directional alignment have been accomplished[3]. Several groups have reported culture of cardiomyocytes under conditions of perfusion using continuous and cyclic loading to mimic *in-vivo* hemodynamic loading[4]. Mechanical stretch simulating hemodynamic loads are studied using technologies like Flexcentral Flexercell® Strain - FX-4000 Flexercell Tension Plus (TM) which consists of a flexible membrane on which cardiomyocytes can be plated and then be subject to oscillatory deflections using a vacuum or pressure source. Electrophysiological studies utilize systems like IonOptix Myocyte Systems where cardiomyocytes grown on a glass slide are stimulated with an electrical impulse and analyzed for contractility via fluorescence using hardware and software developed by the manufacturer. However, to the best of our knowledge the μCCM is the first system that recreates all aspects of cardiac mechanical loading (stretch, shear and pressure) with an integrated circulation network.

The μCCM is designed to culture cells on a thin membrane which can be subjected to mechanical stretch via pneumatic actuation. Further, fluid flow in a circulation network delivers culture medium at flow rates that correspond to different levels of shear stress. The pressure in the chamber is a direct consequence of the flow rate and mechanical stretch. Preload and afterload conditions can be dynamically varied by adjusting the pump flow rate or manipulating the

fluidic resistance of the channels in the circulation network. Each of these parameters can also be varied independently to create any combination of loading conditions. Preliminary studies to date have accomplished design, fabrication, and culture of NRCMs within the μCCM. NRCMs were cultured under static conditions, continuous flow and pulsatile flow. Culture conditions however involved low flow rates (5 μl/min, shear stress < 0.5 dyne/cm^2) and minimal stretch (10%). Apart from differences in contractile function and a slight increase in size, NRCMs cultured in each of these configurations showed little difference in terms of structure and function.

II. Materials and Methods

A. Prototype Fabrication

The basic setup for culture of NRCMs consists of 4 structural layers assembled together to form the cell culture and circulation network (Fig 1.). All 4 layers are made of (poly)dimethylsiloxane (PDMS), a transparent, flexible silicone using soft-lithography techniques[5]. Layer 1 is 2mm thick and consists of a PDMS channel network bonded to glass for external manipulation of the thin membrane on which the cardiomyocytes are cultured. Membrane stretch can be accomplished using pressure, vacuum or a combination of both. Layer 2 is a thin PDMS layer ~ 7μm in thickness which is bonded irreversibly to a 1 mm thick PDMS layer containing cell culture wells 5 mm in diameter. Layer 4, also 2mm thick is aligned and bonded to the Layer 3 is also made of PDMS and contains 50μm X 50μm fluidic channels that make up the circulation network. The thin PDMS membrane (Layer 2) is accessible via through holes that define the cell culture chamber in Layers 3 and 4. Each device consists of 4 culture wells resulting in 4 replicates per experiment.

B. Cardiomyocyte Seeding and Culture

Once the devices are fabricated and assembled, sterility is achieved by steam autoclaving at 180^0 C. The bottom of each cell culture well is treated with a mixture of fibronectin (50 μg/ml) and laminin (50 μg/ml) at 37^0 C for 24 hrs to aid attachment of NCRMs. Alternatively, ECM proteins can be patterned as lines on the thin membrane using a recessed PDMS structure (20 μm gaps) at 37^0 C for 24 hrs, followed by treatment with pluronic to inhibit cell attachment between the patterned lines[6]. Following adsorption of ECM proteins, the wells are washed with 1X phosphate buffered saline (PBS) to remove any unbound proteins. Neonatal rat cardiomyocytes were suspended in DMEM supplemented with 5% FBS, 1% penicillin/streptomycin, 0.1% vitamin B12, 1% BRDU and plated in the culture wells at a seeding density of 5 X 10^6 cells/ml. Cells were cultured in 5% CO_2 incubator and allowed to attach for 1 hr. Additional medium was added to ensure sufficient nutrient delivery. Following 48 hrs in culture NRCMs attach, spread, establish cell-cell contact and begin spontaneous contractions. BrdU is used for the first 48 hrs to induce apoptosis of cardiac fibroblasts and endothelial cells. At this point cells are either maintained in static culture with periodic (every 24 hrs) medium replacement (static controls) or subject to fluid flow and mechanical loading (continuous flow or pulsatile flow).

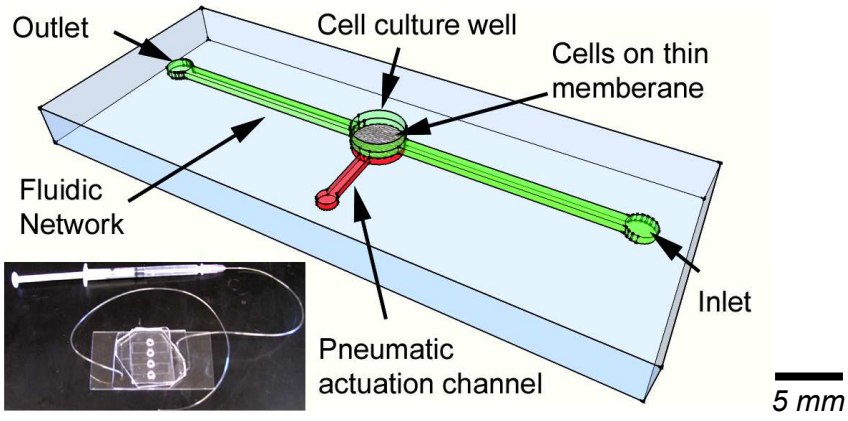

Figure 1: *Simplified schematic representation of the different layers (and components) of the μCCM and* ***(Insert)*** *Actual fabricated μCCM in a 4-well configuration and integrated fluidic network. A Syringe is connected to the inlet and access tubing is connected to the outlet.*

I. Results

A. Contractile Function

Cells in all three configurations were observed under an inverted microscope and the number of contractions per minute was determined. Though cells in all three configurations contracted synchronously, the beat rate varied for each technique. The NRCMs in static culture averaged 30 bpm, where as those under continuous flow averaged 52 bpm and the cells under flow and pulsatile stretch matched the frequency of the membrane stretch at ~ 80 bpm. These results indicate that fluid flow and mechanical stretch play a role in modulating contractile function.

B. Cell Size and Hypertrophy Marker Evaluation

Live images were acquired using a digital camera attached to the microscope. Three images were taken for each well and three or four wells were set up per group (Fig 2.). Multiple images per group were randomly selected and the area of all cells in that image (at least 30 cells/image) was measured using the Metamorph software. NRCMs cultured using each configuration were fixed with 4% PFA and permeablized using tween20 and then stained with fluorescently labeled antibodies for muscular hypertrophy marker: *α-smooth muscle actin (α-SMA*) and sarcoplasmic hypertrophy marker: *phosphorylated phospholamban (Ser 16)* (Fig. 3.)using confocal microscopy as described by Schaub et al[7]

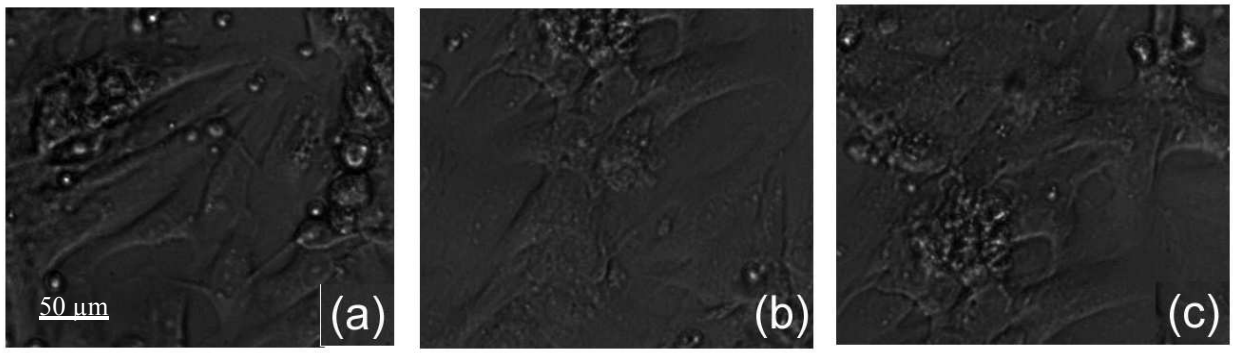

Figure 2: *Phase contrast images showing NRCM cell size in following 24 hr of culture under* ***(a)*** *static,* ***(b)*** *continuous flow (larger) and* ***(c)*** *pulsatile flow conditions (largest). Differences however were small.*

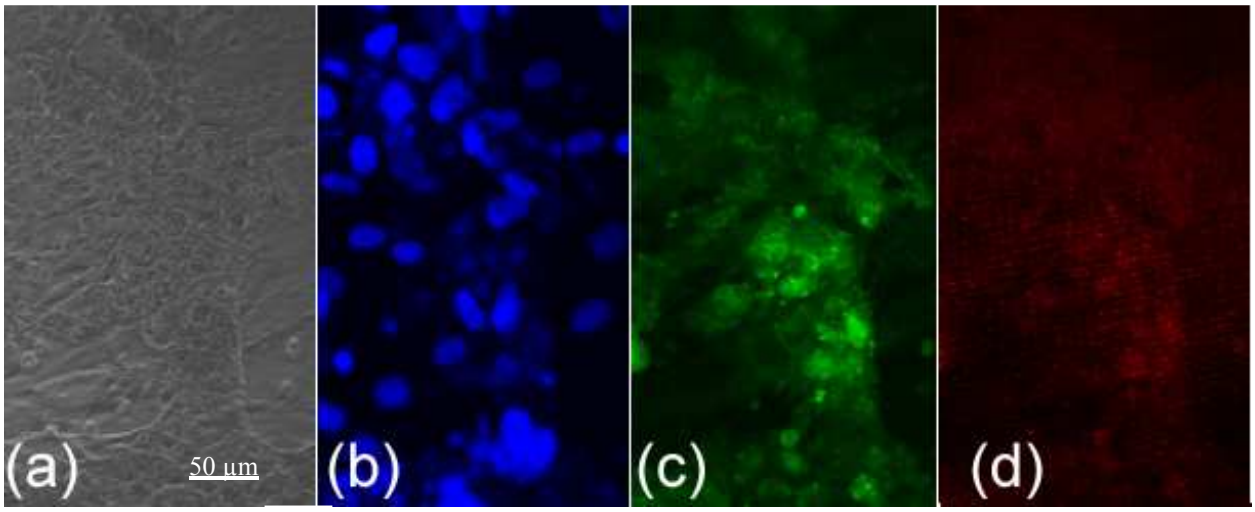

Figure 3 *Staining for hypertrophy markers. There was no noticeable difference between the three groups. Shown is the sample perfused using pulsatile flow.* ***(a)*** *phase contrast,* ***(b)*** *DAPI nuclear stain* ***(c)*** *α-SMA and* ***(d)*** *phosphorylated phospholamban (Ser 16)*

I. Discussion

CVD is the leading cause of death in the United States and claims more lives each year than the next 4 leading causes of death combined[8]. Understanding the molecular basis of manifestations of CVD like myocardial infarction, ischemia, hypoxia, myocarditis, endocarditis, hypertension, cardiomyopathy etc, requires multi-scale, multi-level approaches. Isolated cardiomyocytes are an important preparation for studying cardiac mechanics, as they are free from connective tissue and endothelium. In addition, sub-cellular mechanisms can be assessed in greater detail in single cardiomyocytes as opposed to intact tissue preparations. However, controlling the mechanical environment of intact cells has been a challenging task fraught with intrinsic difficulties of related techniques as they fail to adequately mimic the *in-vivo* environment. The proposed system effectively stimulates cardiomyocytes with *in-vivo* -like mechanical stresses in a circulation network to develop complex models to study cardiomyocyte signaling and mechanotransduction. Information generated from this system bears significantly higher physiological relevance in comparison to existing systems and addresses a critical need in the development of relevant *in-vitro* cardiomyocyte models to identify molecular targets for further validation in animal models and treatment of CVD. Further, this system

can also be used as a platform to study adult cardiomyocytes under field stimulation and cardiomyocyte regeneration using progenitor cells or adult cardiomyocyte cell cycle re-entry.

Towards this long term goal, we have developed a Microfluidic Cardiac Circulation Model (μCCM) that integrates mechanically loaded cardiomyocytes with fluid flow and a circulation network. This system, fabricated using multi-layered (poly)dimethylsiloxane (PDMS) structures consists of a cell culture region on a flexible thin (~ 7 μm) membrane, at the bottom of a 5 mm diameter well. Fluid flow in the network is induced using an external peristaltic pump. The membrane can be cyclically manipulated using pressure, vacuum or both, resulting in pulsatile stretch. Cells patterned and cultured on the thin membrane are therefore subject to *in-vivo* like mechanical stretch via 3D deflections. Preload and afterload conditions can also be dynamically varied by manipulating the pump flow rate and the flow resistance of the microfluidic circuit to represent different hemodynamic loading conditions. Preliminary studies demonstrate the ability to culture and evaluate neonatal rat cardiomyocytes (NRCMs) using the μCCM.

II. Conclusion

This article therefore demonstrates our efforts towards development of an in-vitro cardiac circulation platform for functional cardiomyocyte studies where critical aspects of the in-vivo environment are accurately replicated.

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