Development of a bi-layer heart-on-chip for testing human cardiotoxicity of drugs

Research report

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by

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Statutory Declaration

"I declare in lieu of an oath that I have written this report myself and that I have not used any sources or resources other than stated for its preparation. I further declare that I have clearly indicated all direct and indirect quotations."

Datum: 01.04.2015

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Abstract

The advancement of microengineering approaches has facilitated implementation of a new wave of microfluidic *in vitro* models that mimic the physical and chemical microenvironments of living, human organs. These “organs-on-chips” are 3D devices lined by extracellular matrix and living human cells, perfused microfluidically to provide a complex biochemical and fluid-mechanical microenvironment not possible with conventional 2D or 3D culture systems. Organs-on-chip are suitable for demonstration of physiological and pathophysiological conditions while investigating the molecular mechanism of action, thereby advancing pre-clinical drug discovery and development.

We show the development of bi-layer heart-on-chip that mimics vascularization, maintains the unique anisotropic structure of myocytes and provides both contractile and electrophysiological readouts; all in one microdevice. Our primary goal was to implement an endothelial layer in the heart-on-chip, including design, build and test fluidic set up for culturing endothelial cell and establish seeding and culturing procedure in chip. We assembled the human endothelial layer with human cardiac muscular thin films, and the design and function of bi-layer heart-on-chip was validated in on-instrument experiment. Viable and function tissue could be maintained up to 6 weeks with stable endothelial barrier function and increase in cardiac muscle contractility.

The complex hierarchical structure and highly anisotropic alignment of native cardiac tissue is crucial from tissue engineering view. We also suggest an optical micropatterning technique for cardiac tissue engineering that improves accuracy, speed, and flexibility of patterning and allows for industry scale application. We compared the sarcomeric alignment resulting from optical micropatterning, micromolding and unpatterned isotropic tissue, where results indicate UV patterning is a potential method for replacement of micromolding used for patterning of muscular thin films.

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List of Abbreviations

ECM	Extracellular matrix
2D	Two dimensional
3D	Three dimensional
MTFs	Muscular Thin Films
mTG	Microbial Transglutamase
µCP	Microcontact printing
PDMS	Polydimethylsiloxne
FN	Fibronectin
NRVMs	Neonatal Rat Ventricular Myocytes
OCR	Oxygen consumption rate
BTHS	Barth syndrome
TAZ	Tafazzin
OOC	Organs-on-chip
PK	Pharmacokinetics
PD	Pharmacodynamics
iPS	Induced Pluripotent Stem
ICAM 1	Intracellular Adhesion Molecule 1
EDRF	Endothelium-derived relaxing factor
EC	Endothelial cell
AFM	Atomic force microscopy

1 Introduction

Since heart failures are leading cause of death worldwide, the pharmaceutical industry put great effort into the development of new drugs for cardiac diseases, but they mostly fail in expensive clinical phases due to unforeseen toxicity or lack of human efficacy (Arrowsmith & Miller, 2013). The cardiotoxicity and side effects cannot be predicted with the current technologies; since the commonly used 2D, 3D and animal models cannot faithfully mimic the human metabolism, absorption, excretion and action of given drug.

Three dimensional cell culture models address the limitation of 2D models, by providing an artificially created microenvironment that is more similar to the in vivo condition. Allowing cells to interact with their surroundings leads to enhanced expression of differentiated functions and improved tissue organization, thus providing better understanding how the cells and tissues behave in vivo. 3D models have been very useful in understanding the molecular basis of tissue function and singnaling pathways, nonetheless, they also have backdraws. These systems fail to recapitulate tissue-tissue interaction, the mechanical action of the environment and drug activities (Bhatia & Ingber, 2014).

Cardiac tissue engineering takes advantage of similar mechanical and chemical properties of hydrogels to the in vivo extracellular matrix (ECM). With microfabrication techniques, like microcontact printing and micromolding cell shape and tissue specific differentiation can be controlled to maintain the anisotropic structure that is crucial for myocytes. The lifetime of the cell culture can be extended up to 5-6 weeks by using soft hydrogels instead of stiff substrate, which is long enough to reveal potential cardiotoxicity of drug candidates.

Taking advantage of microfabrication techniques combined with microfluidics lead to the development of the organs-on-chip model. By overcoming the above mentioned limitations, the microfluidic chips lined with human cells and continuously perfused with nutrients, offer valuable possibilities in predictive human physiological and diseased models, also serves as a robust tool for drug discovery.

After reviewing the general microfabrication techniques - such as microcontact printing and micromolding-and their applicability to cardiac tissue engineering, the

report introduces the current stage of organs-on-chips, particularly focusing on the development of heart-on-chip microfluidic system.

1.1 Structure of cardiac muscle

To successfully engineer cardiac tissue, first the hierarchical organization and complex 3D structure of the cardiac muscle (Figure 1) must be understood.

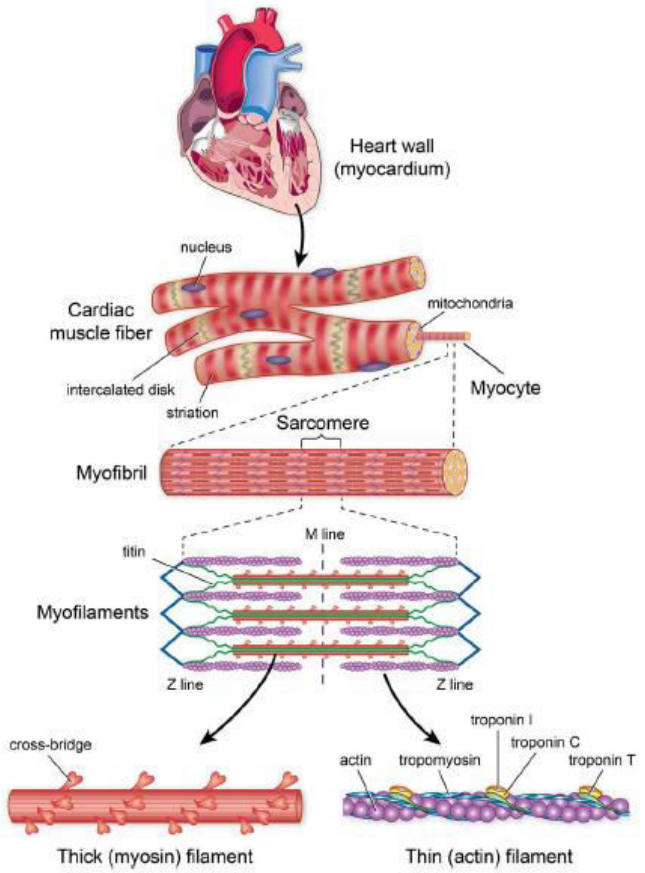

Figure 1 Sarcomere structure. Sarcomeres composed of repeating units f thin and thick filaments, held by together by the protein titin, which behaves like a spring during contraction.. The globular actin proteins are polymerized into filaments, to which tropomyosin and troponin bind. The primary protein of thick filaments is myosin, which provides a a rod-like structure with globular cross-bridges. Adopted from (Olob, Oss, & Hesler, 2014).

The heart is a delicate, yet robust organ, consisting of highly organized cardiac muscle, connectives, tissue, blood vessels and nerves (Parker & Ingber, 2007). The cardiac ventricular wall tissue, which is crucial for the heart's pumping function, consist of approximately four layers of cardiac muscle fibres. The helical wrapping of the differently aligned laminar layers provides a unique anisotropic structure. (Parker & Ingber, 2007). To recapitulate the anisotropic structure is a key element of cardiac tissue engineering, though the most challenging. Cardiomyocytes are generally 10-15 µm in diameter and the length of 100 µm. The sarcomeres of myofilaments are the basic contraction units and they also provide the structural integrity of the entire cardiomyocyte. Sarcomeres are repeating, highly aligned units, composed of thin (actin) and thick (myosin) filaments. The interaction between the motor proteins result in a change in sarcomeric length, thus leading to contractile shortening. The contraction of the cardiac muscle is Ca^{2+}-dependent and the misregulation of calcium ion channels can lead to heart failure. The cardiomyocytes are mechanically and elec-

trically connected to each other in both transverse and longitudinal direction, forming a quasi-continuous electromechanical syncytium. This complex tissue architecture is maintained by the collagenous ECM, whose importance is described in the next chapter.

1.2 Hydrogels as extracellular matrix network

In addition to tissue architecture, mechanical properties of the three-dimensional cellular microenvironment plays a key role in muscle functionality and differentiation (Agarwal et al., 2013) The cardiac extracellular matrix (ECM) network is mainly composed of collagen, but adhesive glycoproteins (e.g. fibronectin, laminin) and proteoglycans help to maintain the tissue structure and architecture. The mechanical coupling of myocytes in transverse direction through collagenous connections register sarcomere Z-lines across the cell membrane, thereby providing slippage free equal stretching(Parker & Ingber, 2007). ECM also directs myofibrillogenesis by providing directional cues for the cardiac myocytes. Besides the physical coupling of the different cells found in the heart, the ECM lattice provides a parallel network for regulatory information transmission, which happens in the form of mechanical forces. These forces are focused on the cell surface receptors integrins that anchor myocytes and surrounding connective tissue cells to a common ECM scaffold (Ruoslahti, 1991). These flexible network is stiff enough to balance cell generated contractile forces and ensure the higher order tissue organization (Parker & Ingber, 2007).The cardiomyocytes are sensitive to the surface chemistry, topology and mechanics of ECM, therefore these key features have to be considered in any artificial biomaterial used for cardiac tissue engineering.

In gel-based cultures ECM is substituted with hydrogels composed of a 3D hydrophilic network that swells in the presence of water thereby supporting cell adhesion, growth and communication. Both physical and chemical properties of the hydrogels can be tuned, therefore they are promising materials for tissue engineering.

Hydrogels are available from arrange of natural and synthetic sources, including collagen, gelatin, fibrin (Pedron et al., 2011), alginate (Khalil & Sun, 2009), and poly (N- isopropylacrylamide) (PNIPAAM) (Fujimoto et al., 2009).

Gelatin, which is a natural polymer recovered from collagen by hydrolysis, has shown the greatest potential for our cardiac application. There are several varieties of gelatin depending on the source of collagen (mainly cattle bones and porcine skin) and the hydrolytictreatment. The enzyme microbial transglutamase (mTG) is used as a crosslinking agent to stabilize the gelatin hydrogel. The stiffness of the native cardiac tissue can be mimicked, by adjusting the gelatin concentration which correlates to the compressive elastic modulus (Young's modulus) in a linear fashion. The Young's modulus for hydrogels with 5%, 10% and 20% w/v is 24 kPa, 56kPa and 114kPa, respectively, and does not depend on the mTG concentration (McCain et al., 2014). The target load for injectable scaffold muscle is 10-30 kPa to exactly match the native conditions. In vitro models provide a flexibility in elastic modulus, which is necessary for the ease of handling and micropatterning, therefore 10% gelatin is used for microfabrications. Furthermore, gelatin can be micropatterned, as discussed in next chapter, in order to align the cardiomyocytes.

1.3 Microcontact printing

Microcontact printing (µCP) which originated from gold patterning, commonly used in a wide range of fields, including the patterning of metal, organic solvents, and,in particular, biological materials. The basic idea of µCP was described by Whiteside group in 1993; PDMS stamp is used to transfer fibronectin (FN) onto the cell culture surface in the same shape fabricated on the surface of the stamp. Fibronectin controls the globular shape of the cells and provide suitable surface for cell adhesion.

µCP represents a simple patterning technique to control the cellular microenvironment, thus cell alignment. Based on the micropatterned spatial distribution the cardiomyocytes orientate their myofibrillar architecture as a response to the geometry of the ECM, in both single-cell and tissue level. PDMS has low surface energy that facilitates the ease of removal of the stamps after patterning, without smearing the ink.

Microcontact printing demonstrates that 2D cultures can induce similar tissue specific differentiation compared to the three dimensional gel embedded configuration,

the only criteria is a microfabricated adhesive island that control shape (Huh et al., 2011).

1.3.1 Shape dependent cellular control

Microcontact printing allows us to culture single cells on fibronectin coated ECM islands in various shapes, surrounded by non-adhesive regions. The cells exert traction forces and spread only until they reach the edge of the ECM island (Singhvi *et al.* 1994; Chen *et al*. 1997; Parker *et al*. 2002). Cells can be maintained in various shapes (e.g. triangle, square, diamond) in the presence of soluble hormones and growth factors (Parker, Ingber, 2007).

Change in the degree to which the cells can physically flatten and distend, can lead to a switch in cells fate (Singhvi *et al.* 1994; Chen *et al*. 1997; Dike *et al.* 1999). Highly spread and flattened cells are proliferating, cells spread to intermediate levels are differentiating, and round and retracted cells indicate apoptosis. Parker et al. showed that the geometric shape also controls cell motility. It was demonstrated by culturing various cell types on polygonal ECM islands that contains corners (e.g. squares, triangles).

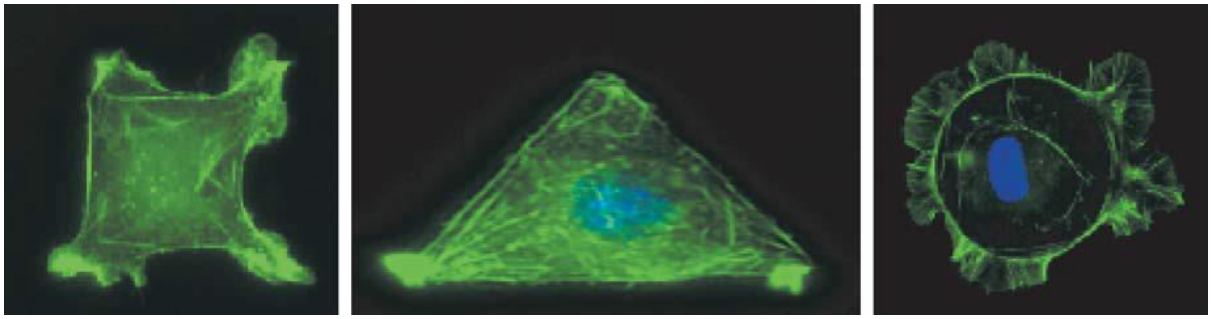

Figure 2: Microcontact printed shape and function relation. Capillary endothelial cells are cultured on a, square (40x40µm), (b) long edge (80 µm) triangular and (c) circular (40µm in diameter) fibronectin coated ECM islands. Cells were stained for nucleus (blue) and for actin filaments using Phalloidin (green).Cell extend lamellipodia from the corners on (a) square and (b) triangular shape (preferentially from acute angel), but no directional bias is observed in (c) circular island (Parker et al 2002; Brock et al. 2003). (Images adapted from Parker & Ingber, 2007)

When the cells were cultured together with motility factors, new motile process were observed, mainly extending lamellipedia, filopodia and microspikes from the corner regions of the island. Brock et al. showed that the lamellipodia extend more likely from acute angle than from obtuse angle. In contrast, circular islands do not exhibit directional bias (Figure 2).

1.3.2 Cardiomyocytes patterned on ECM islands

The Holtzer's model (Dlugosz et al. 1984) and the Sanger model (Dabiri et al., 1997; Rhee et al., 1994) describe the myofibrillogenesis along the length of a sarcomere, whereas micropatterning can be used to control myofibrillogenesis on cellular level, thereby providing new insights into the functional organization of cardiac tissue. Square shaped single cardiomyocytes align their actin fibres in diagonal direction. This actin fibres serve as guidance for sarcomerogenesis initiating from the perinuclear region towards the cell periphery (not shown, Parker & Ingber, 2007). Myofibrillogenesis following the actin stress fibres alignment results in two axes contraction with diagonal orientation. The newly forming sarcomeric Z-lines tend to be perpendicular to the

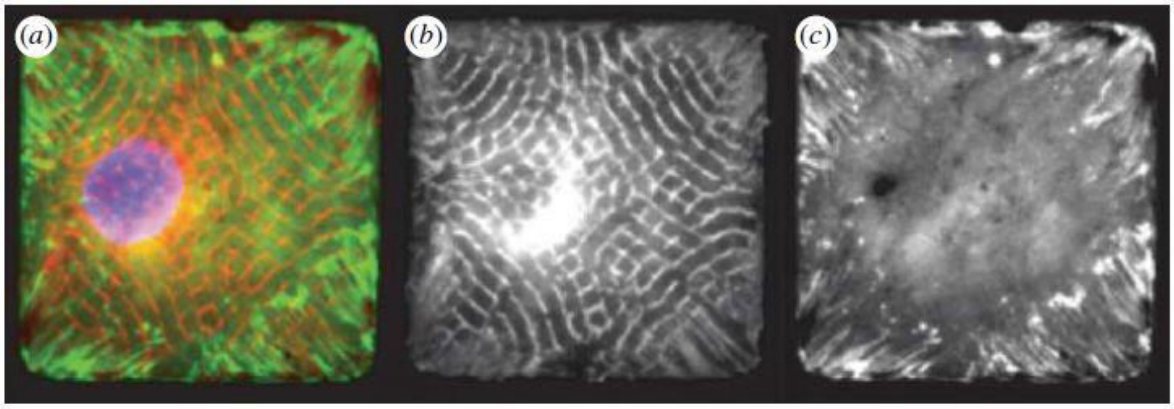

Figure 3 The correlation between deposition of new fibronectin fibrils and the reorganization of myofibrillar architecture within a single cardiomyocytes. Cardiac cells cultured on 50x50 µm fibronectin islands were immunostained and (a) merge image shows the position of the cell nucleus (blue), fibronectin (green) and sarcomeric α-actinin (red). (b) Sarcomeric α-actinin stain reveals the the self assembly of sarcomeric Z-lines are perpemdicular to the cell periphery and rotate through the internal angles of the corners.Sarcomered form two axes of contraction. (c) Fibronectin is concentrated in the corner region, where the traction forces are exerted. (Adapted from (Parker & Ingber, 2007)

the cell periphery, therefore they always rotate through the internal angles of the corners of the square shaped myocytes (*Figure 3*; Parker & Ingber, 2007). The fibronectin fibrils also accumulates and exerting traction forces in the corner regions as it was previously was shown in non-muscle cells in the chapter "Shape dependent cellular control".

1.4 Micromolding for engineered cardiac tissue

Micromolding, a similar approach to µCP, is another way to generate aligned tissues by generating topological surface features that provide physical boundary cues instead of the protein (chemical) cues used in µCP.

1.4.1 Engineering anisotropic cardiac tissue

Highly aligned structure can be observed when NRVMs are cultured on surface features consisting of 10 µm wide ridges spaced by 10 µm wide gaps. The cardiomyocytes spontaneously assemble into anisotropic structure along the linear gelatin features. Sarcomeres with Z-lines are registering perpendicular to the long axis of the myocytes, enabling coherent tissue contractions. Gap junctions are formed between the cells, thus allowing the spontaneous, as well as the electrically paced propagation of action potentials (Parker & Ingber, 2007).

When comparing the alignment of NRVMs cultured on plain gelatin vs micromolded gelatin, McCain et al. verified that the cells are elongated and aligned on the micromolded gelatin hydrogel (*Figure 4*). The differences in myofibrillar architecture across the different tissue types was quantified by extracting orientation information from fluorescently labeled a-actinin in the sarcomere Z-disks (Feinberg et al., 2012), and gave almost three fold higher value for micromolded tissue in comparison to isotropic tissue.

Thus gelatin hydrogels are favorable substrates for cardiac tissue engineering, since the anisotropic structure can be recapitulated without the addition of adhesion proteins.

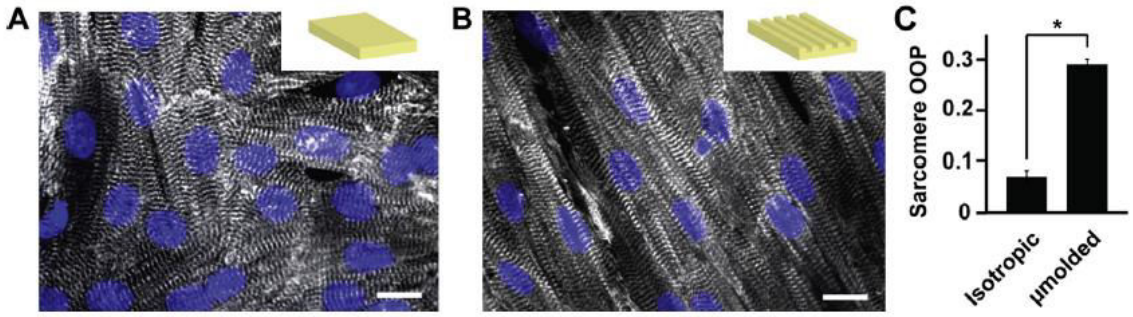

*Figure 4 Anisotropic cardiac tissue on micromolded gelatin. NRVMs cultured for four days on (A) flat and (B) micromolded gelatin for isotropic and anisotropic tissue, respectively. White stains show α-actinin, blue the nuclei. Scale bar is 10 µm. (C) Tissues on micromolded gelatin has higher orientational order parameter (OOP) compared to plain gelatin. Bars represent standard error, n = 4 for isotropic, n = 5 for micromolded, * indicates p < 0.05. Adapted from* (McCain et al., 2014)

1.4.2 Lifetime of microengineered cardiac tissue

Cardiac myocytes are highly sensitive to mechanical stimuli and changes in stiffness and extracellular proteins can alter the phenotype (McCain et al., 2014). Taking advantage of the biomimetic chemical –and mechanical properties of gelatin

hydrogels, the cells can survive longer on micromolded gelatin* compared to fibronectin - PDMS constructs. A measure of the healthy cardiac phenotype is the spontaneous contraction (Marino et al.,1987)(Dranka et al., 2011), therefore McCain monitored over four weeks the percentage of spontaneously beating cardiac cells cultured on micromolded gelatin and on PDMS that was microcontact printed with FN. On PDMS the number of spontaneous contractions continued to fall, such that by the end of the four weeks only 40% of the cells showed healthy spontaneous function, mainly because of delamination and loss of striation. In contrast, 80% of the cardiomyocytes grown on gelatin were viable by the end of the culturing period.

There are several difference between these two culture substrates that contributes to the extension of culture lifetime of engineered myocytes.

The first major advantage of gelatin lies in its elastic moduli as hydrogels are much softer than PDMS. In order to promote the maturation (Jacot et al., 2008) and maximize the contractility output (Engler et al., 2008) the culturing substrate needs to match the physiological elastic modulus of native tissue. The second advantage is, that gelatin is a derivative of collagen, which is the major dominant extracellular protein in the native cardiac tissue. Furthermore, gelatin features (~10µm) are easy to micromold, providing more three-dimensional topography, that leads to an improvement in sarcomerogenesis, cell-cell coupling and conduction velocity (McCain et al., 2014).

The primary neonatal rat ventricular culture mainly consists of cardiac myocytes, but also contains a fraction of fibroblasts. The stiffer PDMS substrate facilitates attachment and proliferation of fibroblasts, resulting in overgrowth and delamination of the cardiac cells in long term culture (Nguyen et al., 2012). The softer hydrogel substrate mitigates these issues, enabling long term culture and thus enabling the screening of drugs that demonstrate cardiac toxicity on a chronic timescale.

1.4.3 Mitochondrial function of engineered cardiac tissue

Cardiomyocytes have higher metabolic activity then other cell types. In rats, human and other mammals over 20% of the cellular volume consist of mitochondria (Schaper et al.,1985). Mitochondria supply myocytes with ATP for physiological function, but it also serve as a reserve capacity, which allows ATP generation above the basal level. In stress situation the cardiomyocytes can quickly adapt to the required higher energy demand.

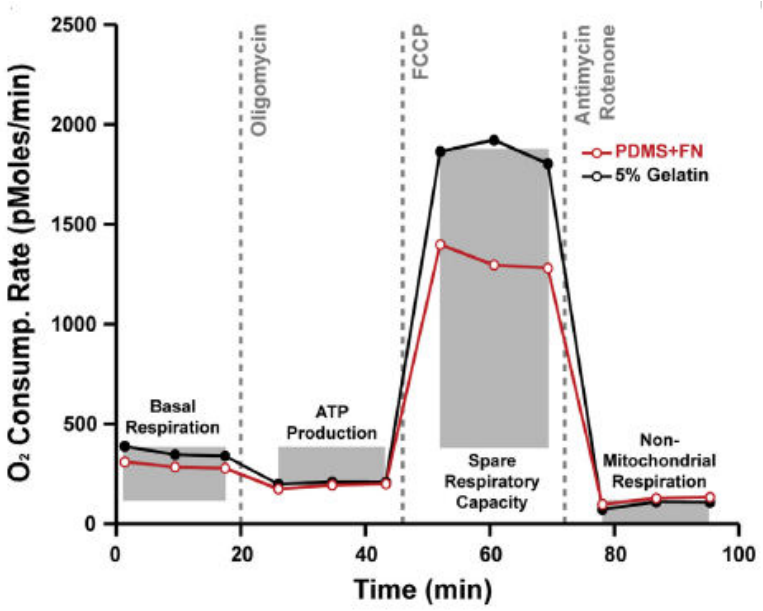

Figure 5 Metabolic differences in microengineered cardiac tissue on gelatin and PDMS. Experimental measurements of oxygen consumption rates for single representative isotropic neonatal rat cardiac tissue at the baseline and after addition of mitochondrial function altering compounds, like Oligomycin, FCCP, antimycin and Rotenone.(Adapted from McCain et al., 2014)

Cardiomyocytes cultured on gelatin have higher spare respiratory capacities than those grown on fibronectin-PDMS as shown by McCain (2014). To determine the spare respiratory capacity, first measuring the baseline oxygen consumption rate (OCR) is measured with an extracellular flux analyser. This is followed by the addition of oligomycin that inhibits ATP synthase, therefore lowering the OCR. Then, adding the uncoupling agent carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone (FCCP) causes OCR to increase to its maximum by depleting the mitochondrial membrane potential. The difference between the base line respiration and the maximized OCR after FCCP injection indicates the reserve capacity of the myocytes. The base line OCR is normalized by the OCR from non-mitochondrial sources, which was determined by the addition of antimycin and rotenone. These molecules inhibit the electron transport chain, thus preventing mito-

chondrial oxygen consumption. As shown in Figure 5, the average basal OCR and ATP production was slightly higher on PDMS, but significant differences can be observed in spare respiratory capacity. Cardiomyocytes cultured on gelatin can utilize higher bioenergetics reserve in stress situation, which leads to improves health and survival.

This study shows that remodelling the extracellular matrix could cause changes in metabolic changes observed in non-physiological conditions: during heart failure the spare respiratory reserve is used up because of the increased oxygen demand (McCain et al., 2014) (Gong et al., 2003).

1.5 Muscular Thin Films

Micropatterning of 2D tissues mimics the laminar anisotropic alignment of native cardiac tissue, but does not provide information about the major measure of heart function, the contractility. Muscular thin films (MTFs) consist of micromolded cardiac tissue cultured on thin gelatin hydrogel film (Figure 6) Cantilevers are laser engraved into the thin film, thus providing a 3D readout during cardiac contraction (Agarwal, Goss, Cho, McCain, & Parker, 2013). Multiple parameters are used to calculate the stress generated by the tissue: the radius of curvature, thickness and elastic modulus of gelatin and the length of each cantilever (McCain et al., 2014).

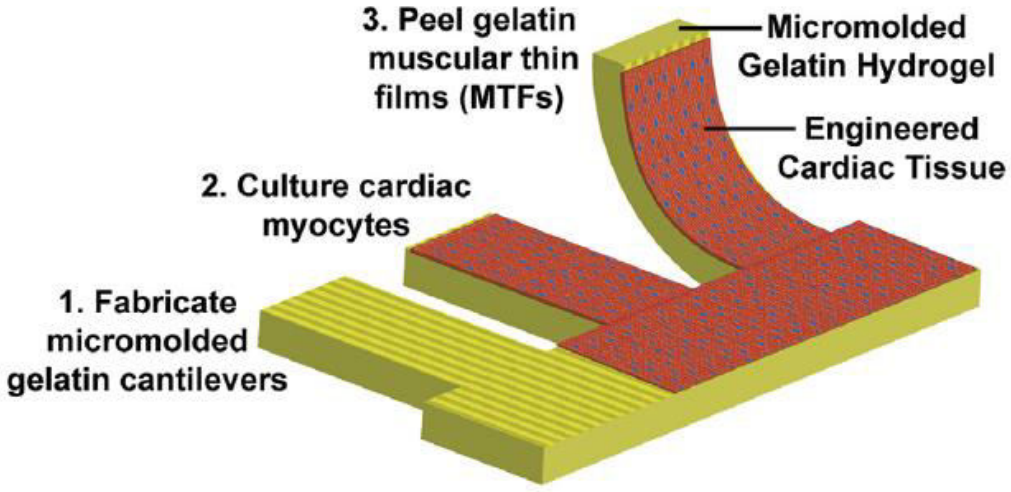

Figure 6 Schematic of fabrication and function of muscular thin films. The MTFs consist of two layers: the micromolded gelatin hydrogel and the anisotropic tissue forming cardiomyocytes on top. He peeling of the cantilevers allows free bending for contractility assay.

1.5.1 MTF contractility assay

After four days in culture the length-wise patterned neonatal rat cardiomyocytes are strong enough to perform contractility assay. The contractile stress is calculated by (Feinberg et al., 2012). Field stimulation electrodes are placed on the MTFs and are paced with 2-5 Hz. In diastole the MTF are relaxed whereas during peak systole (*Figure 7*) the freely bending side of the cantilever curls up normal to the plane of the dish. MTFs also allow measuring the development of any diastolic tension during cell maturation. The sideways curving of the cantilevers refers to the diagonal cell alignment resulting from manual micromolding. The contractile stress is determined by calculating the traction force necessary to deflect the MTFs (Feinberg et al., 2007). A detailed analytical model was developed by Alford et al (2010). The contractile stress directly depends on the cellular architecture: the stress generated by the myocytes is higher when the cells are aligned in the longitudinal direction of the MTFs, providing an increase in sarcomeric z-disc alignment.

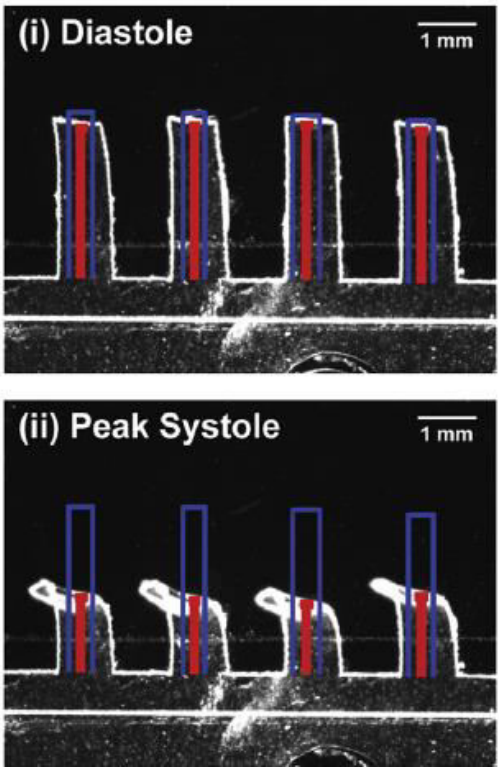

Figure 7: Contraction of Muscular Thin Films. Gelatin MTF cantilevers with neonatal rat cardiac cell cultured on top are relaxed during (i) diastole and bended up in (ii) peak systole. Note that the sideway curling is a result of misalignment of cardiac tissue. Adapted from (McCain et al., 2014).

MTFs enables the direct calculation of contractile stresses generated in a 3D culture, therefore being a key element of the heart-on-chip platform which allows structure-function studies and dose response measurement for pharmacological studies.

1.5.2 Modeling mitochondrial myopathy of Barth syndrome on muscular thin films

Studying human disease pathogenesis by using iPS cells has been hampered by the lack of *in vitro* models that faithfully reproduce disease pathophysiology. Barth syndrome (BTHS) is a mitochondrial disorder caused by the mutation of gene encoding tafazzin (TAZ) (Houtkooper et al., 2009), which often leads to cardiomyopa-

thy in patients. Wang *et al*. could replicate the contractile pathophysiology of BTHS in tissue constructs by combining patient derived, genetically engineered iPS cell derived cardiomyocytes and tissue engineered MTFs. When electrically stimulated, BTHS engineered myocardial MTF cantilever showed significantly lower peak systolic stress compared to controls, indicating the successful recapitulation of diseased condition. The same tissue construct when treated with TAZ modRNA restored contractile strength to a level comparable to those controls, thereby proving that these engineered muscular thin films can model disease correction. The same study also suggests that the impaired contraction in BTHS occurs despite normal myocardial energy stores. Loss of contractile strength can be explained with the defect of sarcomere assembly resulting from TAZ mutation, which could be restored with TAZ modRNA treatment. (Wang et al., 2014).

1.6 Organs-on-chips

The advancement of microfabrication techniques and material science has given birth to the field of microfluidics, the facilitating technology responsible for ushering a new area of *in vitro* models that mimic the physical and chemical microenvironment of living organs, organs-on-chips. The micrometer-sized chambers of organs-on-chips are lined by extracellular matrix (ECM) and living human cells, and perfused microfluidically to provide a complex biochemical and fluid-mechanical microenvironment. The aim is not to engineer a whole living organ, rather create minimal functional units that are able to mimic tissue-and organ level functions. Varying complexity may thus be realized: from the simplest, containing a single microfluidic chamber of one cell type, to highly complex with two or more channels connected and communicating through a porous membrane with multiple cell types mimicking tissue-tissue interfaces. Incorporating physical forces, such as cyclic strain, mechanical compression, fluid shear stress lead to enhanced differentiation and function of many cell types, while high-resolution, real time imaging of molecular events can be carried out (Bhatia & Ingber, 2014).

1.6.1 Microfabrication techniques

The creation of microfluidic devices for organs-on-chips is based on the microfabrication technique known as soft-lithography. In soft-lithography, an elastomeric stamp with patterned relief structures on its surface is used to generate patterns or structures varying from 30nm to 100µm (Xia and Whitesides, 1998). Historically, poly(dimethylsiloxane) (PDMS) is used as the elastomer as it is optically transparent, has a tuneable Young's modulus (typically around 750kDa), among other favourable properties. Examples of soft-lithography include micro-contact printing (µCP), replica molding and micro-transfer molding (µTM), where the generalized soft-lithographic process of making the microfluidic channel, the essential component of the organ on chip, may be seen below (Figure 8)(Shin et al., 2012).

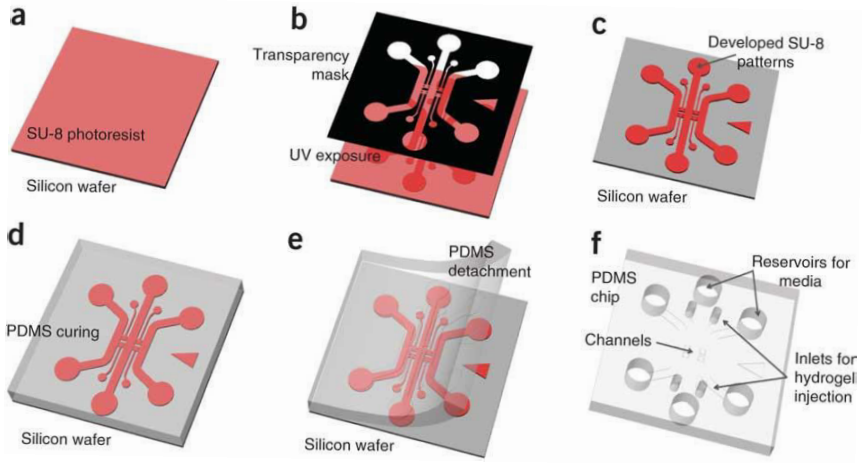

Figure 8: Schematics of the photolithography (a–c) and soft lithography (d–f) procedures. (a) SU-8 is spin-coated and prebaked on a bare wafer. (b) With a transparency photomask (black), UV light is exposed on the SU-8. (c) Exposed SU-8 is then baked after exposure and developed to define channel patterns. (d) PDMS mixed solution is poured on the wafer and cured. (e) Cured PDMS is then peeled from the wafer. (f) The device is trimmed, punched and autoclaved ready for assembly (Schematic adopted from Shin et al., 2012).

1.6.2 Microfluidics and the control of various parameters

One of the great advantages of microfluidic system in contrast to 3D static cultures or bioreactors is that multiple system parameters may be controlled simultaneously. Microfluidics can be identified by the fact, that one of channel dimension of the device is smaller than 1 mm. At this scale, the Reynold's number is typically low (<10), therefore viscous forces dominate and laminar flow occurs inside the channels, allowing for precisely controlled gradients and fluid shear stresses, achieved

by altering flow rate or channel dimensions. Computational fluid dynamic models are used to optimize device geometry and to manipulate nutrient and drug delivery. The microfluidic networks constantly provide the nutrients for the cell engineered in the devices, but also allow the rapid and continuous perfusion of drug solutions and signalling molecule, which facilitates the recapitulation of diseased conditions and also makes prediction of pharmacokinetics (PK) and pharmacodynamics (PD) properties of a drugs. Organs-on-chips are suited for experiments using any cell type amenable to cell culture, but most experiments were carried out using primary human cells. Use of stem cells, especially iPS cell has a great potential in creating diseased models with patient-and disease-specific cells, essentially leading to personalized 'humans-on-chips' (Bhatia & Ingber, 2014).

1.6.3 Organ function and disease models on chip

Organs-on-chips may make it possible to investigate the basic mechanisms of organ physiology and disease. Biological processes that rely on tissue architecture and perfusion, and also short time span pathophysiological processes, are good candidates for the use of organs-on-chips. Only a few organs will be discussed in detail below but to-date, researchers have fabricated chips for the study of the liver, kidney, intestine, lung, heart, smooth and striated muscle, fat, bone, skin, blood vessels, nerve, blood-brain barrier and cancer.

Intestine or "gut-on-a-chip"

This organ chip is a very important model for drug screening. When orally administrated, drugs are mainly absorbed by the small intestine and then diffused through two barriers: a mucous layer and the epithelial cell layer of the intestinal wall. The intestine on a chip is a complex model and takes into account several features: cellular composition (mainly enterocytes and goblet cells), structural features (villi and mucus) and dynamic features (intestinal movements, called peristalsis). Kim and Ingber (2013) found that when human intestinal Caco-2 (enterocyte-like) epithelial cells are cultured under physiological conditions, like peristalsis-like motions and flow, they differentiate into multiple intestinal cell lineages and spontaneously regenerate intestinal villi containing basal proliferative crypts, thus reconstituting

many features of normal small intestine physiology (Figure 9). In addition, the researchers were able to grow common intestinal microbes inside this organ on chip.

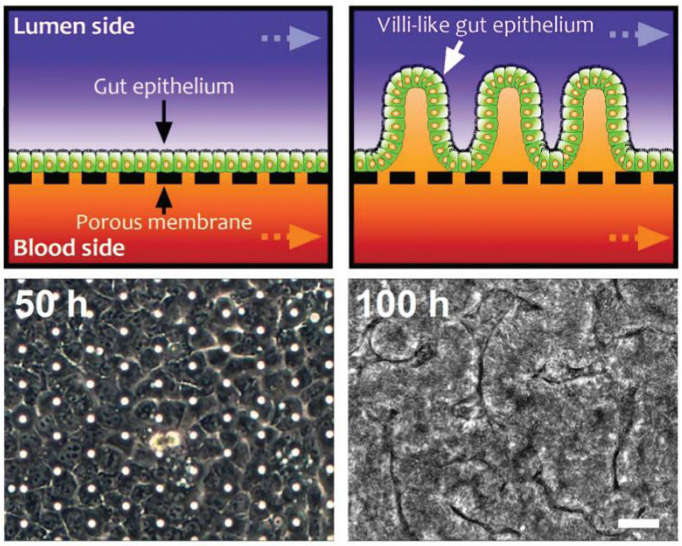

Figure 9: Formation of intestinal villi by Caco-2 cells within in Gut-on-a-Chip cultures. Schematic showing transformation of a planar intestinal epithelium into villus structure (top) and corresponding phase contrast images of Caco-2 cells that undergo similar villus morphogenesis recorded at 50 and 100 h.

Lung on a chip

The lung on a chip is a biomimetic microsystem that reconstitutes the critical structural and functional features of the alveolar-capillary interface of the human lung while also exposing it to breathing motions and fluid flow. This microfluidic device is made of an optically transparent silicone elastomer that contains closely apposed layers of human alveolar epithelial and pulmonary microvascular endothelial cells cultured in two parallel microchannels on opposite sides of a thin (10µm), porous, flexible membrane coated with ECM that separates the channels (Huh et al., 2010). In the upper channel, the alveolar epithelium is exposed to air to mimic the alveolar air space; culture medium is flowed through the microvascular channel; and cyclic vacuum is applied to hollow, side chambers to cyclically stretch the tissue layers (10% cyclic strain at 0.2 Hz) and thus reproduce physiological breathing movements. Re-

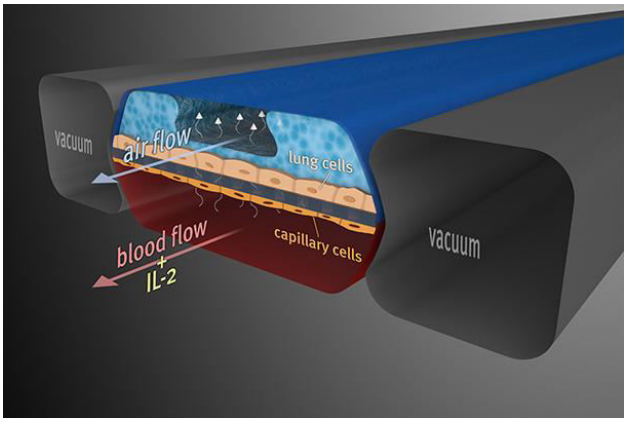

Figure 10: Schematic of Lung-on-chip as model for pulmonary edema

cently, the lung-on-a-chip was used to develop a drug toxicity–induced human pulmonary edema model on-chip (Figure 10) (Huh et al., 2012).
This model effectively reproduced the intra-alveolar fluid accumulation, fibrin deposition, and impaired gas exchange that have been observed in living edematous lungs after 2 to 4 days of therapy with the anti-cancer drug, interleukin-2 (IL-2) in humans. When adding TNF-α or bacterial cells to the alveolar channel, a swift response in endothelium could be observed. ICAM-1 was upregulated and neutrophils were recruited and underwent diapedesis. After migrating to the upper channel, the immune cells engulfed the living bacteria, thus providing proof of principle for using a biomimetic microdevice to create a human disease model-on-a-chip that can be used to study human cell responses to clinical therapies.

Cancer on a chip

One of the major problems in the field of oncology today is that same cancer in the same patient can often respond differently to the same chemotherapeutic agent depending on whether it is located at its primary location or at a distant metastatic site. Multiple *in vitro* microfluidic models have been developed to investigate the mechanisms of cancer development as well as the metastatic cascade, including study of tumor spheroids, tumor-stromal interactions, angiogenesis, hypoxia and intravasation under flow (Chen et al., 2011; Hsiao et al., 2009; Kim, Lee, Chung, & Jeon, 2013; Sung et al., 2011; Zervantonakis et al., 2012). Although these models have advanced cancer research and increased our understanding of the tumor microenvironment, none of these *in vitro* models permit analysis of how cancer cells differ in their response to therapy depending on their unique, *organ-specific* microenvironment. The Organ-on-a-Chip approach, which allows one to recreate different types of human organ microenvironments (e.g., lung vs. liver), enables development of *in vitro* cancer models that mimic organ-specific tumor responses to chemotherapy. This Organ-on--Chip modeling approach is exciting because it employs relevant human cells, overcomes the disadvantages of current cancer model systems and provides entirely new capabilities. Examples of novel capabilities of these devices include modulation of the mechanical microenvironment of the organ (e.g., breathing in lung, flow in the vascular) that are necessary for normal organ function and required for modeling drug delivery dynamics. Also, tu-

mor-stroma, tumor-endothelium, tumor-epithelium, and tumor-immune cell interactions all can be manipulated independently, visualized at high resolution in real-time, and quantitated in a highly controlled manner. Because the organ-on-a-chip system is 'synthetic', it can be studied in its simplest form (e.g., tumor + endothelium) or when integrated with different types of cells (normal epithelium, fibroblasts, immune cells, EPCs) and/or molecules as desired, and they can be added to either the parenchymal (tissue) or endothelial channels, to add progressive complexity to the system. And as mentioned previously, the organ-on-a-chip cancer model may also be made with human primary cells, iPS cells, or patient-specific cells, a requirement to move towards the realization of personalized cancer therapies.

1.7 Heart-on-chip

Since the heart is one of the most complex organ, the development of the heart-on-chip is particularly challenging. Ideally, the heart chip design mimics vascularization, maintains the unique anisotropic structure of myocytes and provides both contractile and electrophysiological readouts; all in one microdevice.

1.7.1 The design

McCain *et al*. have successfully showed the concept of MTFs and also that the lifetime of the cardiomyocytes can be extended when using gelatin as ECM. These findings serve as strong basic of the heart-on-chip, combining it with microfluidics can be taken to a higher level. Since all the DARPA funded organs-on-chips have to be compatible with each other, some of the criteria e.g. certain dimensions cannot be changed, but the specific needs of cardiomyocytes and the complex purpose of the heart chip also had to be considered. Because of the already discussed advantageous properties of PDMS, every kinds of organs-on-chips, and generally microfluidic devices are made out of

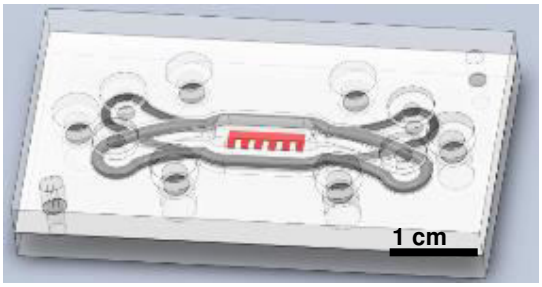

Figure 11: Dual channel heart-on-chip. The chip housing is made out of polycarbonate, and two vapour polished channel facilitates culturing human cells allowing for three types of quantitative readouts: barrier function via endothelial layer, contractile strength by using MTF and electrophysiological by incorporated MEAs (not shown).

PDMS. Since the cardiomyocytes like the gelatin more than the PDMS as culturing substrate, the golden standard has been changed
to gelatin based MTFs. This also means, the heart-on-chip device has to be fabricated in parts and PDMS cannot be used. PDMS known to have excellent air permeability, which also holds a great risk to initiate bubble formation inside the channels. Bubbles are problematic strictly from a fluid mechanics point of view, but also create problems for the cell culture including increased levels of shear, cell delamination, pressure build-up, and no or extremely low-flow conditions preventing nutrient delivery and the build-up of waste. Finally, bubbles that become trapped directly over the cell-culture area of devices can result in patches of massive cell death due to drying out of the cells.
Since the motivation for biomimetic organ devices is to replace current models in pre-clinical studies, therefore the criteria and requirements of pharmaceutical industry also have to be considered during development. Another drawback of PDMS is based on its chemical and physical properties: it absorbs drugs and chemicals. There is no golden standard method yet for the quantification of the amount of absorbed chemical during drug perfusion into the chip channels, therefore most of the pharmaceutical companies clearly reject the use of PDMS. Based on these arguments, the heart-on-chip is made out of polycarbonate, which a strong, durable material with great light transmission, not absorbing chemicals. The fabrication includes milling of the chip housing, vapour bonding the membrane, and vapour polishing of channels for a smooth surface. The bilayer chip design facilitates the culture of two different cell types, thus mimicking tissue-tissue interfaces. The channels are separated by a porous membrane, in the upper channel a confluent endothelial layer is engineered, in the bottom channel the cardiomyocytes are cultured on gelatin based MTFs that contracts, alterntively the cells are cultured on customized MEAs (*Figure 12*).

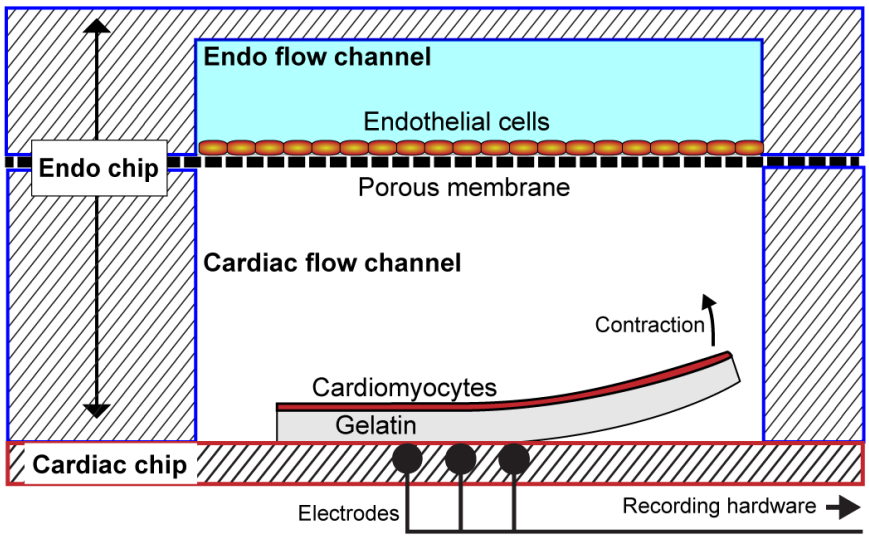

Figure 12: Schematic of heart-on-chip. In the top channel human endothelial cells are cultured on the porous membrane that separates the two channel. Into the bottom channel human cardiomyocytes are cultured on either top of MTFs or on MEAs. Flow is applied to both channel.

At the current stage of the development, MTFs and MEAs cannot be combined, therefore in one chip only two types of readout can be measured at the same time: either barrier function and contractile strength, or barrier function and electrophysiological properties.

1.7.2 The importance of implementing endothelial channel

In the human body the nutrients and oxygen are transported via the bloodstream within the different kinds of organs. In order to facilitate the organ-chip couplings, every DARPA organ-on-chip was designed to have an endothelial layer which tend to mimic vascularization. The parenchymal tissues of every organ require a specific media that is not compatible with other cell types. The endothelial layer in each chips allows for microfluidic chip-to-chip linking via a common media, basically functioning as a selective barrier between blood and tissue. Also, it is beneficial during drug studies: the drug needs to pass an endothelial barrier, which leads to more physiological drug delivery. The signaling events between cardiac endothelium and myocardium remains unclear, therefore this biomimetic device has a great potential in endothelial-cardiac signalling Jerry Smith and colleagues showed that two different messengers are released from endothelial endocardium and effecting underlying myocardium: endocardin has a unique prolonging effect on my-

ocardial contraction, whereas endothelial-derived relaxing factor (EDRF) elicits the opposite effect, but the cellular mechanism is unclear (Gulino-Debrac, 2013). Endocardial endothelium is ontologically similar to vascular endothelium, and unpublished studies of Fort, S and Lewis, M.J showed that the release of EDRF from vascular endothelium has effect on myocardial contraction in the whole heart similar to EDRF released from endocardial endothelium. Endocardin and EDRF exert their effect in systole, therefore likely to influence predominantly relaxation and diastolic filling events which might have pathophysiologycal relevance. Further studies suggest that acute endothelial damage plays a role in certain disease condition, like eosinophilic cardiomyopathy (Smith, Shah, Fort, & Lewis, 1992).

1.8 Optical micropatterning

The excellent biocompatibility and negligible immunogenicity of hydrogels make the an attractive candidate for tissue engineering, however its application is limited due to properties related to mechanical stability and thromobogenicity (Chan et al., 2007). Photochemical cross-linking is emerging technology to improve physiochemical properties of hydrogel, it is faster than using chemical crosslinkers, and also has reduced toxicity compared to other methods. Natural protein crosslinking accelerates with age and leads to stiffer and stronger cornea. Based on this observation, riboflavin (also known as vitamin B_2) is used in artificial corneal crosslinking to treat conditions like keratoconus, where the constituent collagen is prone to enzymatic degradation. The photoinitiator riboflavin induces ECM protein crosslinking, meanwhile preventing the cornea from damage by absorbing the UV radiation (Meek & Hayes, 2013; Snibson, 2010).

Recapitulating the architecture of anisotropic structure of cardiac tissue engineering is a key element. The current patterning techniques, e.g. microcontact printing and micromolding are slow and inaccurate due to manual operation. We suggest a novel micropatterning method for gelatin substrates using UV laser for cardiac tissue engineering that improves accuracy, speed, and flexibility of patterning. Patterning with UV laser on pure gelatin surface results in burns and boils, but when treating the gelatin with riboflavin, a UV sensitive cross linking agent, patterned lines were observed without major damage to the surface. The alignment if cardiac tissue cultured on optically patterned gelatin can be compared to micromolded and

isotropic tissue by quantifying the sarcomeric orientation order parameter, which is an established metric to quantify the mean sarcomeric orientation perpendicular to Z-disk.
Optical micropatterning is a promising method for cardiac tissue engineering both on laboratory and industry scale, and can be incorporated into muscular thin film technology.

1.9 Objective

The primary goal of the research project was to develop an endothelial layer in the heart-on-chip. The first step was to design, build and test flow loop for culturing endothelial cells in heart on-chip, followed by establish a seeding and culturing protocol of endothelial cells in chips. We verified the efficacy of off-instrument flow setup for endothelial culture. We aimed to show viable and functioning endothelial- and cardiac tissue in bi-layer heart on-chip while measuring endothelial barrier transport using fluorescent tracer, at the same time, we try to gain preliminary information about the effect of the present of endothelial layer on muscular thin films during two-week on-instrument experiment.
The secondary goal was to develop optical micropatterning of soft gelatin for cardiac tissue engineering. Gelatin can be patterned with UV laser when using riboflavin, but the method has to be optimized. Both gelatin preparation and laser properties e.g. frequency, power and speed were adjusted and studied with atomic force microscopy in order to reach confluent anisotropic tissue. In this report we compare the sarcomeric alignment resulting from optical micropatterning, micromolding and unpatterned isotropic tissue, where results indicate UV patterning is a potential method for replacement of micromolding.

2 Methods & Materials

2.1 Development of bi-layer heart-on-chip

2.1.1 Endothelial cell seeding and microfluidic culture

The endothelial cells used in this model were green fluorescence protein (GFP) positive human umbilical vascular endothelial cells (HUVECs) (Lonza), and were cultured in EBM-2 (Lonza) supplemented with 5% fetal bovine serum and growth factors according to manufacturer's protocols. For long-term culture, cells with passage 4-5 are ideal, using higher than P7 is not recommended. The cells were maintained at 37°C in a humidified incubator under 5% CO2 in air. Before cell seeding, microfluidic devices, and all the components necessary for manifold based flow setup, were cleaned by sonication in distilled water, sterilized and surface activated by plasma treatment. The porous membrane was coated either with fibronectin (50µg/ml in PBS), rat tail collagen type I (100µg/ml in PBS), or in the mixture of both, and incubated for 3 hours at 37 °C or overnight at 4°C. Next, the appropriate silicone O-rings into the screw slots in the manifold and add in the respective threaded luers (inlets) and barbs (outlets) for the endothelial and cardiac channels. Tight seal is essential for proper seeding and flow loop setup. Successful seeding densities of HUVECs in top channel varies from 2-8 million cells/ml, the described results were achieved with 4 million cells/ml. Through manifold based flow setup the cells were seeded manually with a pipet, but this design also allows for semi-automated seeding, when cell droplets are pulled into channels using peristaltic pump. The endothelial chips were incubated at 37°C, 5% CO_2 for approx. 1 hour, until the cells are attached. The attached cells were then perfused with culture medium by peristaltic pump at a volumetric flow rate of 60 µl/hour. The HUVECs were grown to confluence within 1-2 days, then culture medium was exchanged for maintenance media, a mixture of DMEM/F12 and EGM-2 SingleQuot, containing only 0.5 % FBS (established by Kyung-Jin Jang,PhD, Ingber lab)

2.1.2 Cardiac cell culture

For the optimization of optical micropatterning, neonatal rat ventricular myocytes were isolated from two day old neonatal Sprague-Dawley rats according to protocols approved by the Harvard University Animal Care and Use Committee. After extraction, ventricles were homogenized in Hanks balanced salt solution followed by overnight trypsinization and digestion with collagenase at 4 °C (1 mg/mL, Worthington Biochemical Corp.). Cell solutions were strained and re-suspended in M199 culture media supplemented with 10% heat-inactivated fetal bovine serum, 10 mM HEPES, 0.1 mM MEM nonessential amino acids, 20 mM glucose, 2 mM L-glutamine, 1.5 mM vitamin B-12, and 50 U/mL penicillin, and pre-plated twice to reduce non-myocyte cell populations. Cardiac myocytes in a density of 1500 cell / mm^2 were seeded for each well of 8-well dish containing simple square gelatin-samples. Media was exchanged to maintenance media containing 2% Fetal bovine serum (FBS) in every 48 hours.

Human iPS cell-derived, Cor4.U cardiomyocytes (Axiogenesis) were cultured on the MTF/ MEA channel of heart-on-chip system. One frozen vials contains 1 million 100 % pure cardiomyocytes, which need fibronectin (50 µg/ml) coated area after adding thawing media mixed with puromycin. Cardiomyocytes are extremely sensitive to shear stress, therefore the centrifugation of cell suspension during thawing is not recommended. Mixed cell suspension is gently distributed in 3 wells of 6 well-plate and incubated for 3 - 5 h at 37°C, 5% CO2, and 95% humidity. The thawing media is changed for a mixture of Cor4.U cell culture media and puromycin. After overnight incubation, the cells were cultured only in media, and on the following day the cells are dissociated and seeded on the gelatin layer of MTFs in 2000 cells/mm^2. Prior to assembling of MTF and endothelial layer of heart-on-chip, the human cardiomyocytes were cultured for 1 week on gelatin.

2.1.3 Muscular thin film fabrication

Topas plastic slides for cell culture purpose (75 mm x 25 mm) were covered with low adhesive tape which was cut by LPKF ProtoLaser U3 system in a square shape. The tape is peeled away so that an inner rectangle remains covered. The masked plastic slide was plasma treated in order to increase the adhesion of the gelatin on the unmasked area. Before adding gelatin to the surface, the inner rec-

tangular tapes were removed, providing a non-adhesive area to later enable the gelatin cantilever to lift from the surface.

To make gelatin solution, 18% w/v gelatin (Sigma-Aldrich) was dissolved at 65 ˚C and degassed in vacuum chamber. 8% w/v of cross-linking agent Microbial Transglutamase (Ajinomoto) was prepared and warmed up to 37 ˚C, then equal parts of the gelatin and MTG was mixed, resulting in a final concentration of 9% w/v and 4% w/v, respectively. The gelatin solution was quickly pipetted onto the increased adhesion region of topas slide. To micromold, PDMS stamps with 10 µm lines spaced with 10 µm was gently pressed against the gelatin droplet and cured overnight. The next day, the gelatin was re-hydrated with distilled water and the glass slide was carefully peeled off the gelatin. Cantilevers (1 mm wide, 2 mm long) were laser engraved into the dehydrated micromolded gelatin using an Epilog laser engraving system (Golden, CO) with the following settings: Power 3%, Speed 6, Frequency 2500 (adopted from McCain *et al*). After cutting, the outer border of the tape was carefully removed without disrupting the gelatin cantilevers. An important step in fabrication of MTFs is to pre-peel the cantilevers off the surface by using a tweezers before seeding the cardiac cells, thus avoiding tissue damage later on.

Gelatin chips were sterilized with ultraviolet irradiation, rehydrated in PBS and stored at 4 °C until seeding of human iPS cell-derived cardiomyocytes.

2.1.4 Assembling endothelial flow set up for off-instrument experiments

The individual manifolds component were cleaned with Alconox and/or sonicated in 70% ethanol, while the endothelial chip was sonicated in distilled water. The components were dried with compressed air and sterilized and surface treated with plasma treatment. Every step after sterilization was carried out in cell culture hood. The membrane was ECM coated with either 100 µg/ml rat tail collagen type I, 50 µg/ml fibronectin, or alternatively with the mixture of both. The ECM coated endothelial channel was incubated in the manifold base with fitted PDMS gasket. The appropriate silicon O-rings were loaded in the crew slots and threaded luers (inlets) and barbs (outlets) for both endothelial and cardiac channel were added to manifold. Manifolds were screwed into the chip base, ensuring that the ports at the

base of the manifold line up with the ports on the chip. GFP+ HUVECs in 2-6 million cells/ml concentration were seeded, incubated for 1 hour for attachment of the cells. 5 ml syringes were added to the threaded luer chip inlets and filled with endothelial media containing 5% FBS, as well as 10% FITC-inulin in endothelial channel. Ismatec IPC peristaltic pump were placed into cell culture incubator designated for OOC experiment, and Ismatec tubing with 0.25 mm in diameter were connected to both endothelial and cardiac outlet barbs. The tubings were connected to peristaltic pump and 50 ml Falcon tubes were placed in incubator as outflow collectors. 60µl/hour flowrate was turned on, and the proper sealing and pumping was ensured after 3 hours. Endothelial cells were kept in manifold based flow setup until they reached confluency and they were ready to be coupled with the cardiac channel.

2.1.5 Interrogator: on instrument experiments

The Interrogator is an automated instrument developed by the Wyss institute (Figure 13), which is able to integrate multiple organ-on-chips and designed for ease of "plug-and-play" approach. Each organ-on-chip is assembled onto a universal chip holder cartridge that dimension wise fits every type of organ-on-chip developed by the Institute. More than 20 chip can be integrated at the same time, providing incubator conditions for the cells: 37 °C, 5% CO_2 and controlled humidity and sterile enviroment. The media is pushed through the chips by using pressure driven system. A robotic sampling hand is built in to automate the media exchange and refill and collect samples from the outflow for further testing. Also a microscope is built in the interrogator to check cell viability without unplugging the chips. When using this special automated functions, the

Figure 13: Organ-on-chip interrogator to generate data predicting the human response on chips.

experiment is run in so called “fully automated” manner. Because of the temporary defect of autosampler and the microscope unoptimized for recording MTF contractions, we used “automated manner”: the media is perfused by interrogator and the flow rate is controlled by computer software, but the media refill and outflow collection were done manually and chips were taken off instrument to record MTF contraction with phase contrast microscope. The experiments carried out using interrogator is referred to as “on-instrument” culture, whereas the design of the developed “off-instrument” flow set up is described in results 0 section.

2.1.5.1 Heart-on-chip assembly and mounting on interrogator

First the endothelial and MTF chip compartments were assembled in a cell culture hood, and then the bilayer chips were mounted on the chip holder cartridge and plugged on interrogator by following a fairly tedious and long protocol, which is abridged and briefly described below.

1. Plasma-treatment of cartridge components
   All cartridge and chip components were plasma treated using a plasma cycles established by Wyss microfabrication team.
2. Degasing of media
   Both cardiac and endothelial media was warmed up and degased by using steriflip.
3. Assembly of heart chip (endothelial top and cardiac MTF bottom, Figure 14)
   First endothelial chip was removed from the manifold based flow set-up, as next the heart chip was removed from the seeding well. Cardiac chip was sandwiched between the endothelial layer and the bottom chip base, which had to be clamped with screws.
4. Priming of heart chip
   Once the full chip was assembled, both channels were primed with appropriate media using a pipette.

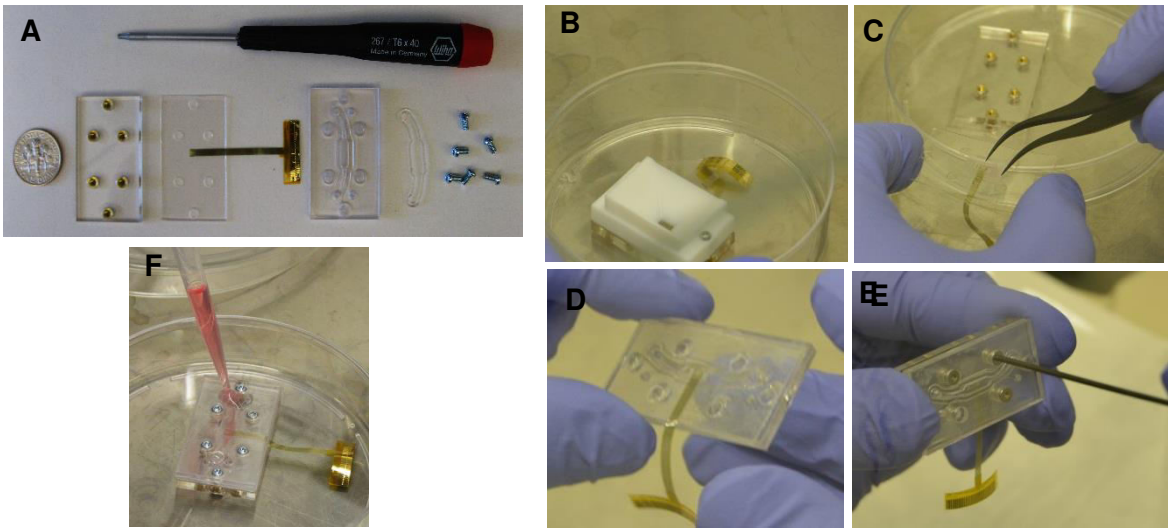

Figure 14: Assembling endothelial- and cardiac chip. A) Shows the individual components of a full, bi-layer heart-on-chip: chip base with threads, seeded MTF or MEA cardiac chip bottom (MEA shown on picture), seeded endothelial chip, PDMS gasket, T6 4 mm screws, T6 torx screwdriver. B-C) Removing heart chip from seeding well, D) placing cardiac chip upside down on endothelial chip. E) Fixing chip base to the bottom cardia chip, F) Priming of both channels with appropriate media.

5. Priming of cartridge (Figure 15)

   The inlet and outlet tubing were built in the universal cartridge. The media from the syringe containers to the chip, and from the chip to outflow reservoir were transported via these tubings. Inlet ferrules and syringes were filled with media, the bubbles in ferrules are dislodged by swirling Pasteur pipette.

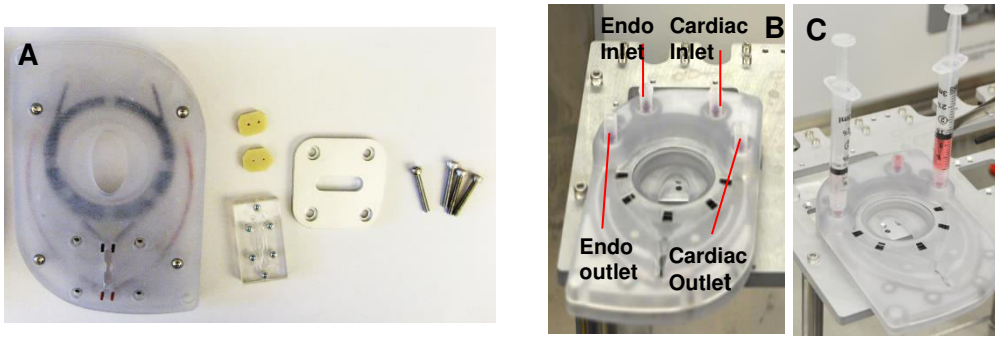

Figure 15: Priming of chip holder cartridge. A) Components necessary for chip mounting: cartridge with custom-cut peek connectors, assembled heart chip, rubber gaskets, metal base plate, screws, Allen wrench (not shown. B) Cartridge showing cardiac and endothelial inlet and outlet ferrules. C) Primed inlets and outlets.

   Syringes were connected to inlet ferrule, and plungers are pushed down until droplets emerge at peek connectors on cartridge bottom, then syringes were removed. The outlet tubing was primed in the same way, but in the end syringes should not be removed, otherwise all the media run out (low resistance).

6. Mounting and clamping of heart chip to cartridge (Figure 16)

   To make fluid-fluid connection, the chip ports and the peek connectors were wetted, then peek connectors were squeezed into chip port until chip bottom is flush

with rubber gasket. Since the heart-on chip is made out of polycarbonate, a metal base was needed to fix the chip to the cartridge. Reservoir syringes with filters were connected on the inlet port, and appropriate media was added (endothelial media+ FITC-inulin; Cardiac media). Although every precaution was implemented to avoid bubble formation during assembly, a final check for bubbles with flash light was necessary.

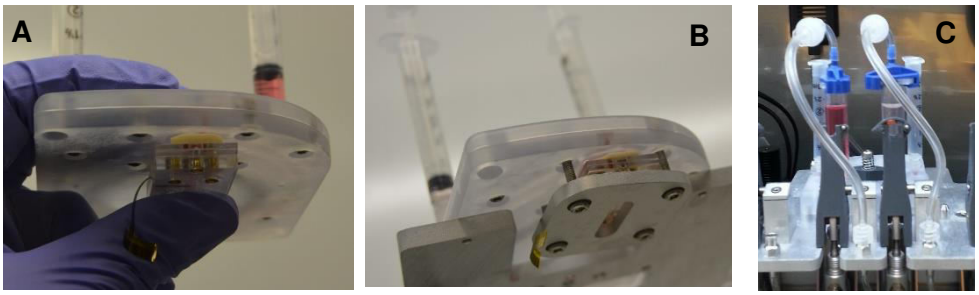

Figure 16: Mounting chip to cartridge and onto interrogator. A) Wetted peek connectors are squeezed into chip ports, B) Chip is clamped with metal base to cartridge, C) cartridge is plugged on interrogator slot and hydrostatic heads are connected.

7. Mounting of cartridge to Interrogator (Figure 16)
   Assembled chip on cartridge was plugged onto the interrogator slots, the hydraulic heads need to be connected and pressure set for desired flow rate.
8. Setting up the flow
   Flow rate was controlled by a computer software, 60µl/hour was set in both endothelial and cardiac channel. The perfusion was automated by interrogator.

2.1.6 Permeability assay

A cell layer forms cell-cell junctions that can inhibit molecules from passing between cells. This barrier can be an important measure of the function of the cell. The permeability of the endothelial barrier was assessed by measuring the rate of FITC-inulin transport from the top human endothelial channel to the bottom cardiac compartment (Figure 17) Inulin (MW 2000-5000, 100 µg/ml

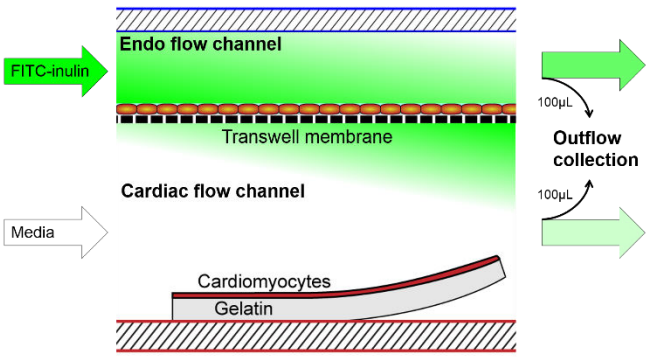

Figure 17: FITC-inulin is introduced to endothelial channel and diffused through the endothelial layer and membrane to the cardiac channel. The transport of tracer is measured by measuring fluorescence intensity of collected outflows.

in endothelial medium) is a small hydrophilic molecule with fast diffusion, minimal uptake and absorption by cells, therefore it is a suitable “neutral” indicator for channel to channel diffusion. The rate of diffusion of the inulin-FITC through the cell layer depends on the tightness of the cell layer; the tighter the layer the higher the barrier function and less inulin-FITC passes into the bottom channel in a given time frame. The inulin-FITC is tagged with fluorescent FITC, and the amount of FITC can be read via plate reader and correlated to the concentration of inulin-FITC in the media, which was used as an index of barrier permeability.

2.2 Optical micropatterning for cardiac tissue engineering

2.2.1 UV patterning

Usually permanox, a thicker type of plastic were covered with tape and laser engraved, and coated with gelatin in the same way as described in MTF fabrication (2.1.3). For the optimization of micropatterning, the MTFs cantilevers were not cut, only a square gelatin sample was used, for control experiment the gelatin was micromolded or left as plain surface. Before UV patterning, the gelatin samples were soaked into Riboflavin that was prepared in a concentration of 0.3% one day prior to patterning and its filtered supernatant was used to treat gelatin samples. The samples were washed thoroughly and dried with air for one hour. The thickness of the samples were taken into account when UV patterning with LPKF ProtoLaser U3. Given the smallest size of the laser beam, the patterned lines are 25 µm wide and spaced by 7 µm (we refer to this as 22 µm pattern). The laser speed was varied from 80-150 mm/sec, whereas power and frequency settings of laser was kept constant, 0.164 W and 50 kHz, respectively. After UV laser cutting samples were observed under a stereoscope and prepared and sterilized for seeding of NRVMs.

2.2.2 Atomic force microscopy

The topography of UV patterned gelatin surface was examined using AFM (Asylum research) in contact mode, using dry samples. The gelatin samples were prepared on permanox slides to avoid the bending or movement of the sample. The

used AFM tip was made out of silicon nitride and had a rectangular shape (RC800PSA, Asylum research).

2.2.3 Immunostaining and image analysis

Engineered neonatal rat cardiac tissues were pre-fixed with 2% paraformaldehyde directly added to the media for 2 minutes, then fixed with fresh 2% paraformaldehyde and 0.5% Triton-X for 10 minutes. Tissues were incubated with primary antibodies against sarcomere α-actinin, DAPI and Alexa Fluor 546 Phalloidin for 60 minutes at 37 °C. Followed by incubation with secondary antibodies against mouse IgG conjugated to Alexa Fluor 488 for 60 minutes at 37 °C for each coverslip 6 fields of view were imaged using a Leica SP5 X MP inverted confocal microscope with 63x/1.3 glycerol objective. The degree of alignment was quantified using the so-called 2D-orientational order parameter (OOP). This parameter is derived by brightly staining the sarcomeric Z-discs of the actin fibrils using alpha-actinin antibodies, followed by image processing that extracts the angles of the z-discs relative to each other. From the distribution of angles, a main direction of the fibrils can be extracted as well as the associated "weight", i.e., the proportion of fibrils actually pointing into that main direction. This weight corresponds to the OOP.

3 Results

3.1 Development of a functioning bi-layer heart-on-chip

The heart-on-chip is fabricated in parts: the endothelial cells are cultured under flow on the porous membrane that forms the bottom of the chip's top channel and the interface to the chip's bottom channel. Well aligned and contractile cardiac tissue is prepared separately and is later mounted below the top channel later where it forms the bottom channel. The myocytes are cultured on top of a gelatin film with movable cantilevers, forming the muscular thin films (MTFs). The MTFs adhere to a plastic slide, which can be clamped to the top channel using a plastic base. In this report, the expression "endothelial channel" is used for the top channel only, "cardiac channel" or "MTF channel" refer to the cardiomyocytes on MTFs. After combining the separate components, the full chip will be referred to as the "assembled chip".

3.1.1 Design, build and test flow set up for endothelial culture (off-instrument)

The endothelial cells cultured inside the channel require nutrients and oxygen, which are provided by continuous perfusion of culture media. The polycarbonate chip itself cannot be connected directly to syringes nor to peristaltic pumps, thus an adaptor, also called manifold necessary to maintain efficient and constant flow. The most important design criteria the manifolds and flow set up have to fulfil are to ensure complete fluidic sealing for leak-free flow and sterility, to provide even flow without bubbles, and to allow for optical imaging of the cells.

In the previous flow loop design the tubing was glued or taped to a metal adaptor that connected the inlet and outlet ports of endothelial chip. This initial set up (**Error! Reference source not found.**a) was tedious to assemble, the adaptors were leaking and bad sealing lead to bubble formation in the channel. The membrane holes are fabricated in an angle thus making the membrane opaque and causing imaging issues. To improve the existing set up, eight chips were joined in

a base and the individual chips were covered with a full length manifold providing connections to tubing (Figure 18b).

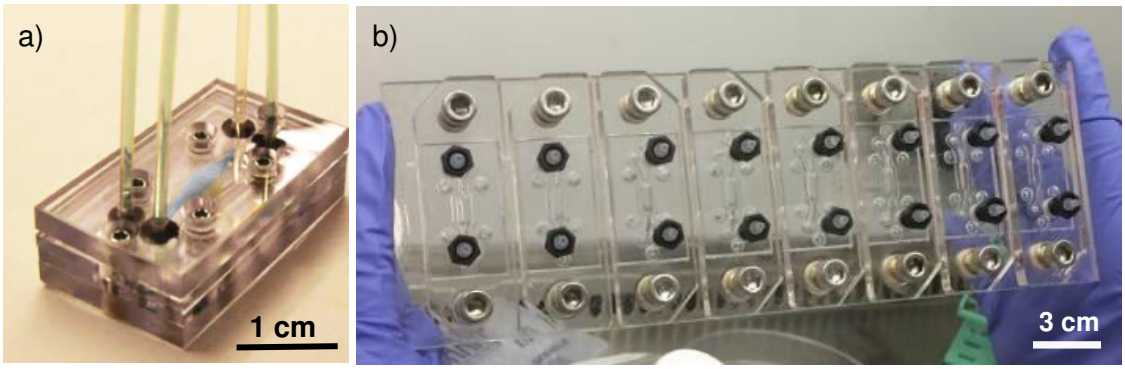

Figure 18: Inefficient flow setups for endothelial cell culture. a) existing single chip setup where the chips are connected to tubes via leaky adaptors. b) joined-chip setup with improved adaptors, but the long assembly leads to cell death. Both designs have uneven membrane and ECM coating, imaging and bubble formation issues.

This joined-chip set up had a lots of important improvements, including switching to a clear polycarbonate membrane with perpendicular pores, which allows for optical imaging and commercially available threaded barbs for achieving a tighter connection to the tubing. Beside the disadvantage that the thick base impedes microscope imaging, the major drawback is that, again, the assembly takes too long: by the time the last chip is connected to the manifold, the cells in first chips start to die. Furthermore, the strategy in which the cells are seeded first and then connected to the manifold fixed lead to pressure differences within the two ends of the channels and air bubbles can be easily introduced. Uneven coating of the membrane with ECM, and the need for separate media reservoirs are common design flaws in both setups shown in Figure 18. Learning from the previous mistakes, the final design consists of a thinner, optically clear manifold base in which the endothelial chip fit snuggly and two separate manifolds connect to the inlet and outlet ports of the channels (Figure 19) O-ring sealings and in house made PDMS-gaskets assure the tight connection between adaptors and manifolds. Syringe media reservoirs can attached to the chip with perforated plungers that allow for adequate air venting. The commercially available barbs can be connected to tubing with a minimal diameter of 1.3 mm, which, when using Ismatec IPC peristaltic pump, results in a flow rate that is 20 times greater than the desired 60 µl/hour. Therefore, we use tube connectors made from the tips of 27 gauge blunt needles to serve as connectors between the larger- and tubing with 0.25 mm in diameter.

This thinner tubing reduces the flow rate to approximately 60 µl/hour. Once the chip manifold is connected to the peristaltic pump, the media is pulled from the inlet media reservoir through both the endothelial

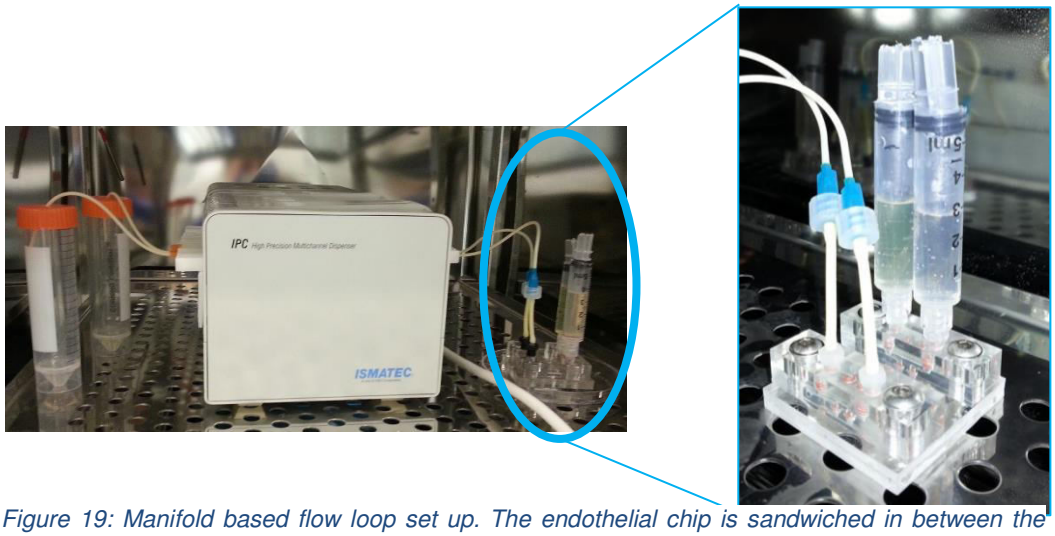

Figure 19: Manifold based flow loop set up. The endothelial chip is sandwiched in between the manifold with adaptors and manifold base. Proper sealing assure the even media flow pulled by the peristaltic pump from the inlet reservoir through the endothelial chip, and the outflow collected separately.

and the cardiac channel (to avoid an open air-liquid interface in the chip) and the channel outflows are collected in Falcon tubes. For further optimization, the cell culture media formulation was adopted from the Donald Ingber lab: the media is warmed to 37 °C 1 hour prior to use and degassed using a vacuum setup, thus reducing the risk of bubble formation in the channels. The fluidic check of the flow setup was done by perfusing food color through the two channels. The test showed no leakage, nor abnormal mixing through the membrane. After seeding, images of endothelial cell layer were taken, showing no/minimal bubble formation even several days in culture. It is important to evenly coat the membrane with ECM proteins to achieve a confluent endothelial monolayer, but that is not possible if the membrane is wavy or rough. To prevent the membrane from buckling and wrinkling, some changes to the chip itself were necessary: vapor-polishing the channels provides a smoother surface that liquids can wet more easily, and vapor bonding of membrane results in a flat membrane. Also, the inlet ports of the endothelial chip are tapered for easier injection of ECM proteins. Further, an optimized plasma treatment protocol was adopted from the Ingber lab, thereby achieving a very hydrophilic, wettable surface of the membrane and also sterilizing the chip

itself, as well as other components. To test the efficiency of ECM coating, Eosin can be used to stain collagen for examination under the microscope.

The membrane was coated with 100 µg/ml rat tail collagen type I and both the endothelial and cardiac channel were flushed with Eosin Y, an acidic red stain. After washing the channels with PBS, the cardiac channel was clear, but the collagen coated endothelial channel remained pink, confirming deposition of collagen. Examination under the microscope showed that the staining agent was equally distributed all the membrane, indicating even coating with collagen. Thus the design changes for optimization of ECM coating was successful.

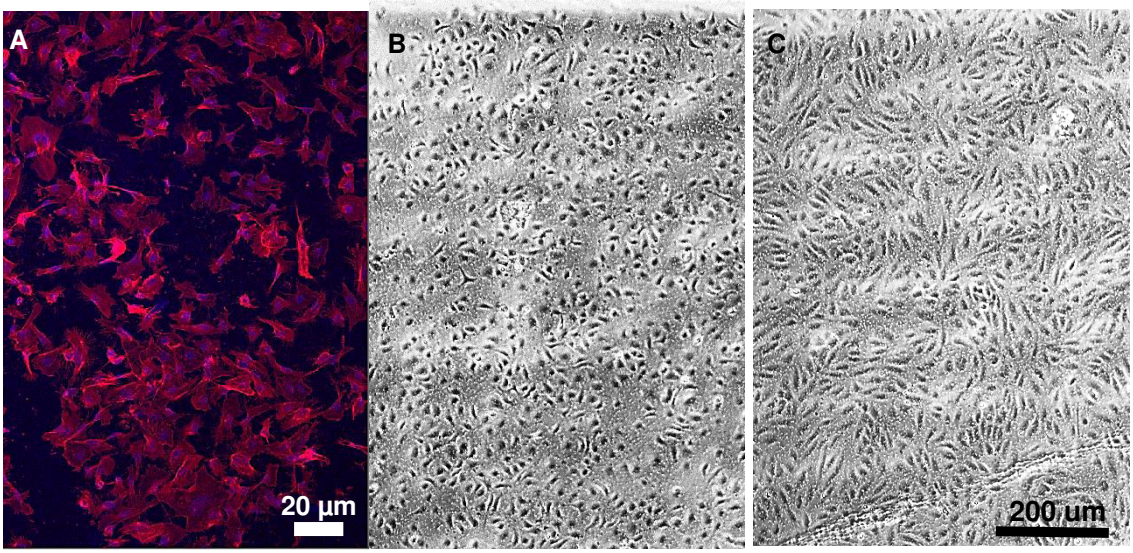

Figure 20: Attachment of Endothelial cells to rat tail collagen type I in chip. A) One hour after seeding HUVECs adhered to ECM well enough to resist flow. DAPI (blue) stains for nuclei, Phalloidin (red) for Actin. B) Phase contrast image of HUVECs one hour after seeding. C) HUVECs reaching confluency after 1 day in flow condition.

3.1.2 Achieving confluent endothelial layer in heart-on chip

After successfully designing, building and testing the off-instrument flow setup, a protocol for seeding and culturing Human Umbilical Vein Endothelial Cells (HUVECs) in the endothelial chip was developed. The protocol consists of the following parts: device and cell preparation, ECM coating, cell seeding and flow set up. This paragraph discusses the findings that led to the establishment of this particular protocol, and a more detailed description of the manual seeding procedure is described under section 2.1.4. One of the specific application of the organs on chips (OOCs) is to test drugs and recreate diseased conditions in long term experiments, thus the endothelial layer has to remain confluent for at least two weeks.

The ECM is not critical only for cardiac tissue engineering, but also plays a key role in vascular biology: besides providing structural and organizational stability, also support signalling events involved in cell migration, proliferation, and survival (Davis & Senger, 2005). Therefore, the life time of the tissue in chip mainly depends on the interaction of endothelial cells and the ECM. As the endothelial cells cannot survive for long without medium replacement in the channel, it is very important to estimate the time point when focal adhesions are formed after seeding, allowing for media perfusion to start. Already one hour after seeding the HUVECs

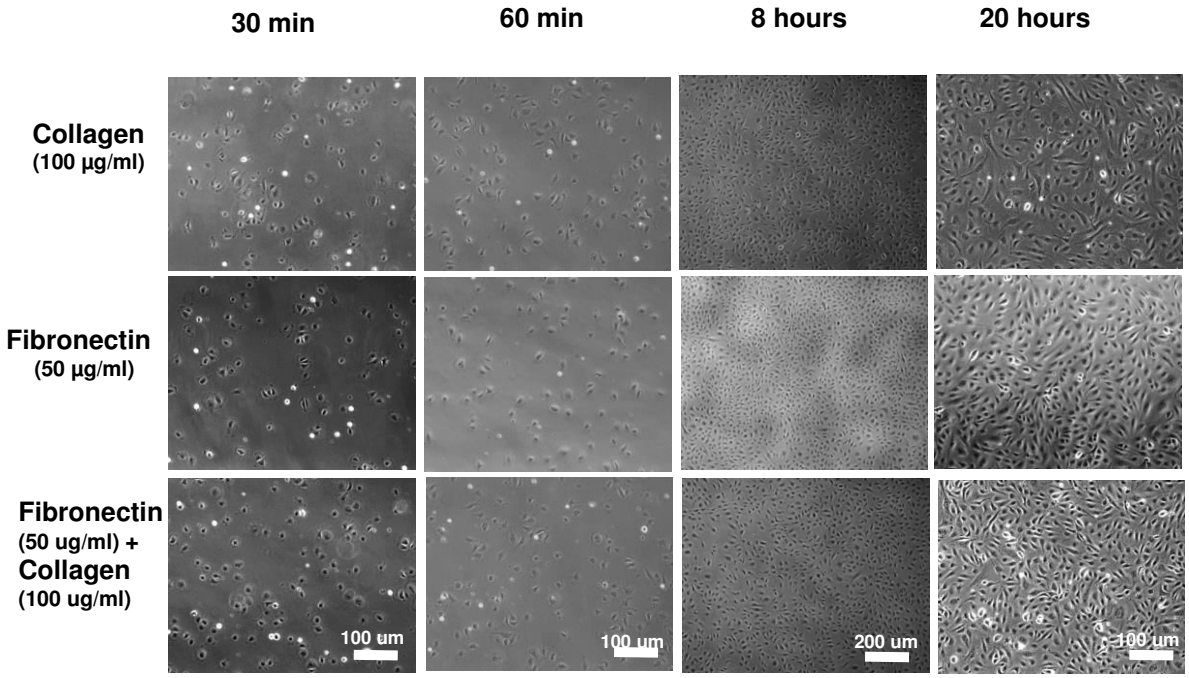

Figure 21: Phase contrast images of HUVECs cultured on different ECM. EC cells were seeded on collagen, fibronectin and on mixture of both, and the cell monolayer and integrity were compared over 20 hours. The presence of fibronectin appears to be beneficial for cell-cell interaction and proliferation.

adhered well to rat tail collagen Type I coated membranes, thus perfusing media containing 5% FBS with 60 µl/hour flow rate after one hour incubation does not shear the cells off. Tissue confluency is reached after one day in culture under flow condition using the manifold based flow setup. The endothelial monolayer was maintained for 4 days under these conditions before placing the chip on-instrument for further culture. Collagen Type I is used as standard ECM coating in every OOC developed by the Wyss Institute. Finding the optimal ECM coating is very important, especially when engineering vascular endothelial tissue and studying signalling through the EC layer in the future. We compared the time necessary to reach tissue confluency in flask and on-chip when using Collage type I and Fi-

bronectin with media containing 5% FBS. Three T25 flasks were treated with different conditions: collagen, fibronectin, and a mixture of collagen and fibronectin (Figure 21). Based on cell morphology examined with phase contrast microscope, in the first 8 hours after seeding, there were no differences in the speed of focal adhesion formation or cell proliferation, but after 20 hours the cells cultured on collagen exhibited a lower density than the cells in the other two flasks, using fibronectin and a mixture of both. This suggests that fibronectin accelerates proliferation and formation of cell-cell junctions. To confirm this preliminary results for on-chip condition, two endothelial channels were seeded: one with collagen, the other with

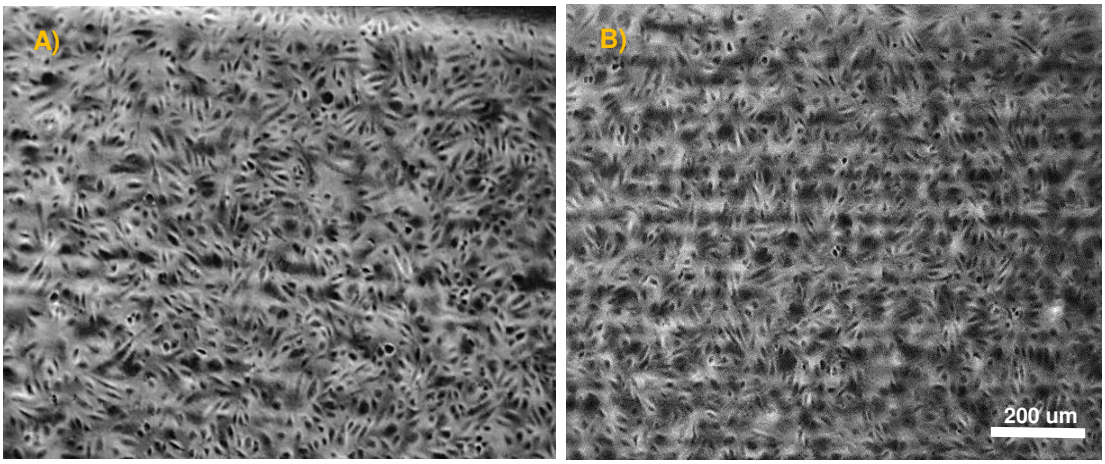

Figure 22: HUVECs cultured on collagen vs fibronectin coated endothelial chip. A) Phase contrast image of HUVECs cultured for 20 hours in 100 µg/ml collagen coated channel. B) Endothelial cells cultured in 50 µg/ml fibronectin coated channel, phase contrast image 15 hours after seeding.

fibronectin. Both chips were seeded at the density of 4 million cells/ml and cultured with media perfused with 60 µl/hour. Based on morphology, within 15 hours the cells on fibronectin reached approximately the same confluency as cells on collagen coated membranes did in 20 hours. To further investigate the effect of using fibronectin or collagen as extracellular matrix for HUVECs, both chips were placed on interrogator to show two weeks viability and barrier function.

3.1.3 Assembled chip on-instrument

Usually after one day in culture the endothelial tissue reached 80% confluency.As full confluency can be expected for the following day, thus the endothelial chip can be removed from the off-instrument flow loop and assembled (following the procedure described in 2.1.5.1) with the separately cultured cardiac MTF and MEA channels. To prove two weeks on-instrument viability of cardiac tissue, the as-

sembled chip was placed on interrogator with automated media perfusion. Daily test of endothelial barrier and cardiac contractile and electrophysiological function were performed.

3.1.4 Verifying endothelial barrier function on interrogator

Once the assembled chip was run in the automated flow mode - because of the complex design of the chip, cartridge and components, and the lack of a suitable microscope with long distance objective - the morphology of the endothelial layer cannot be monitored during on-instrument experiments. The first, simplest indication of the endothelial tissue damage is the color change in outflow media because of the mixing of the cardiac and endothelial media (Figure 23). The daily barrier test, which quantifies the mixing of medium exchange between the two channels, is a quantitative way to measure the health and confluency of endothelial tissue. As a control experiment, a 2 days barrier test was performed on a blank chip, where the endothelial channel was coated with 100 µg/ml collagen, but no cells were seeded. Because of the porous membrane and the 60 µl/hour flow rate, about 36-37% of the FITC-inulin leaked to the cardiac channel (Figure 24). The barrier test could be evaluated on two assembled chips, both with MTFs as cardiac part, but one of the chip was coated with collagen, the other with fibronectin. 5-10% leakage was observed in both of the chips, which is considered as a very tight, healthy endothelial barrier. Initially, the collagen coated chip did not form a full barrier, similar to the blank chips condition, 39.43% of FITC-inulin tracer leaked through the barrier. The cell-cell junction formation was complete after 24 hours and stable 5-6% leakage was observed during 14 days. On Day 3 the endothelial channel had to be pressurized to remove a bubble, which lead to increase of leakage to 20%. The endothelia layer fully recovered until the next day. The fibronectin

Figure 23: Media colors of assembled chip with healthy barrier function. The color of inlet and outlet media of both cardiac and endothelial medium is the same, no mixing ob-

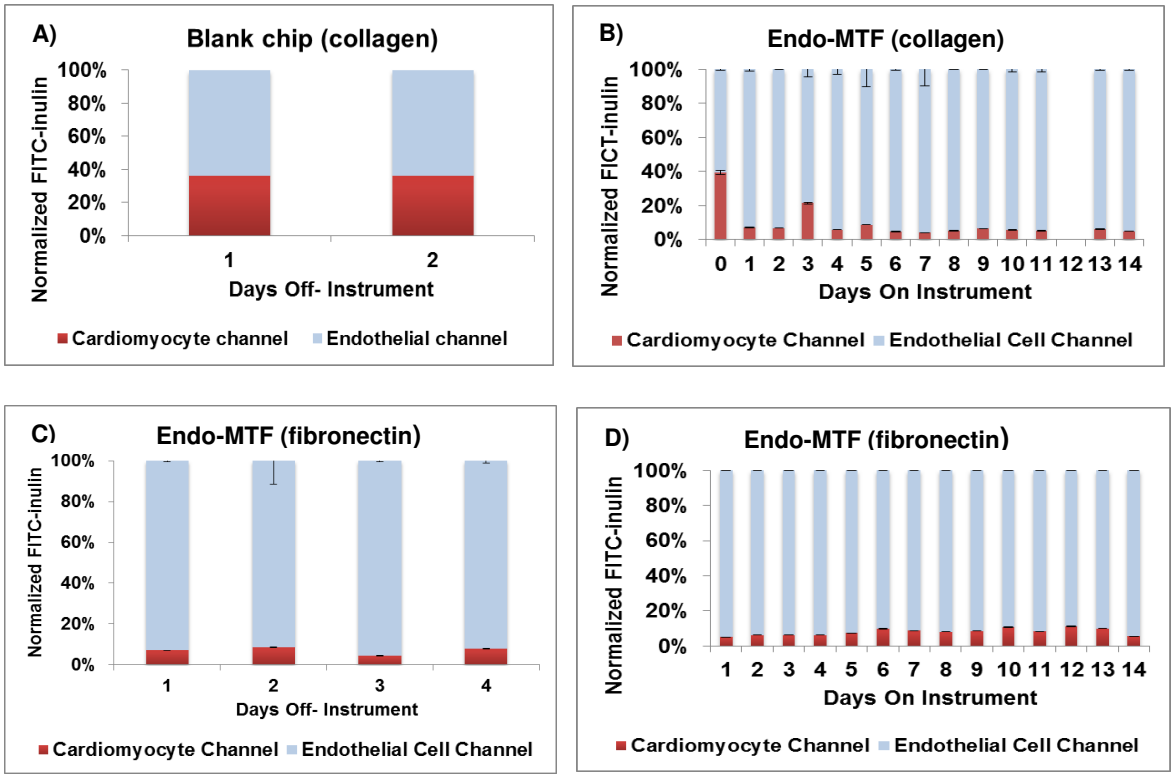

Figure 24: Verifying barrier function of HUVECs in chip. A) blank test on collagen coated channel without cells. B) collagen coated assembled chip on interrogator. Fibronectin coated assembled chips off interrogator (C) and on interrogator (D). Stable 5-10% leakage was maintained over 14 days (B, D).

coated chip was cultured in the newly designed manifold based flow setup through 4 days. The endothelial barrier was fully formed already on the first day, 7.03 % of tracer leaked to the cardiac channel. On day 5 the assembled chip was put on interrogator and endothelial barrier was maintained for 6 weeks when isoproterenol drug studies were performed (results are not shown in this report).

3.1.5 Cardiomyocytes in dual-channel heart-on-chip

Successfully seeded endothelial channels were combined with cardiac channels holding the human cardiac MTFs to form complete, dual-channel heart chips that can be cultured on-Instrument. At the time of assembly, cardiac MTFs were on average 1 week old, i.e., they had been seeded and cultured separately in open-well tissue culture dishes to allow for sufficient attachment, alignment and maturation of the induced pluripotent stem cell (iPS)-derived cardiomyocytes on the gelatin MTFs. In assembled, on-instrument heart chips, both tissue channels were cultured under equal flow rates. The muscular thin film contraction was video-recorded every day during the on-Instrument experiment and the data was ana-

lysed by Janna Nawroth, PhD. The MTF videos were taken at 60 frames per second to capture motion and displacement of the gelatin cantilevers during contractions (Figure 25). From these videos we can extract the spontaneous beat frequency as well as maximal MTF curvature. Further, given that we know the mechanical properties of the gelation cantilevers, their curvature can be converted to peak systolic muscle stress, i.e., the normalized contractile peak force developed by the muscle that pulls the cantilever up and into a curl.

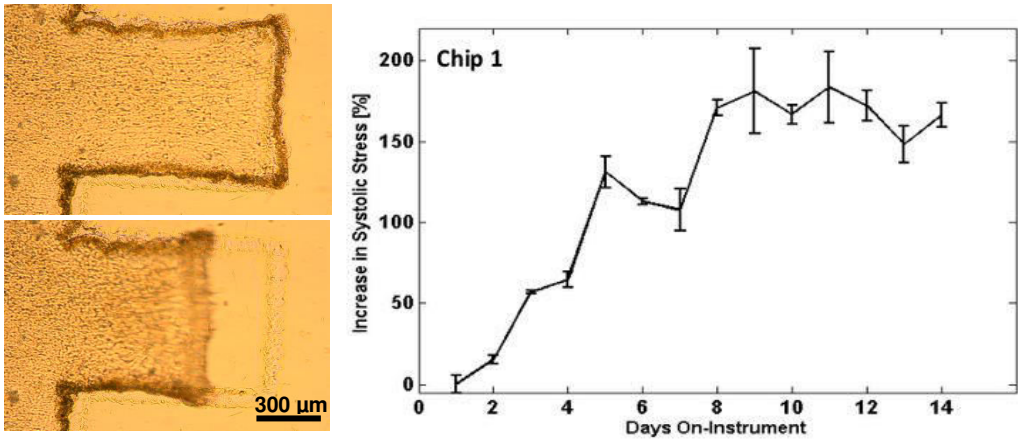

Figure 25: Human cardiomyocytes on MTF in bi-layer heart-on-chip. Snapshots of recorded spontaneous contraction of MTF: A) systolic B) diastolic. Graph shows an increase in systolic stress measured from baseline during 14 days on-on instrument.

The two-week on-Instrument experiment demonstrated that under these conditions the cardiac muscle not only remained viable and functional but that the beat frequency decreased (not shown) and the contractile strength increased substantially (Figure 25). This finding indicates continuous maturation of the tissue (which could involve thickening of cells and improved cell-to-cell connections) and it is consistent with other studies on engineered iPS-derived cardiac tissues (Shinozawa, 2012).

3.2 Development of optical micropatterning for cardiac tissue engineering

To test the alignment of NRVMs on gelatin resulting from optical micropatterning, three different conditions were compared: isotropic, micromolded and UV patterned. The micromolding is the golden standard which provides the highest sar-

comeric alignment, whereas isotropic means a completely random sarcomeric organization achieved without any pattering techniques.

The NRVMs were seeded on gelatin chip that is UV patterned and the trenches are spaced 22 µm from each other using optimized laser parameter (frequency, power and speed). The immunostained images showed that cardiomyocytes were not well aligned, the sarcomeric OOP was 0.175 which is in the range of the isotropic sarcomeric OOP. Furthermore, when looking at a bigger field of view, the tissue was discontinuous and gave the impression that there were physical obstacles that changed the directions of the cells. We prepared gelatin samples in the same way but without seeding cells and the topology of the UV patterned gelatin was examined using atomic force microscopy (AFM) (Figure 26).

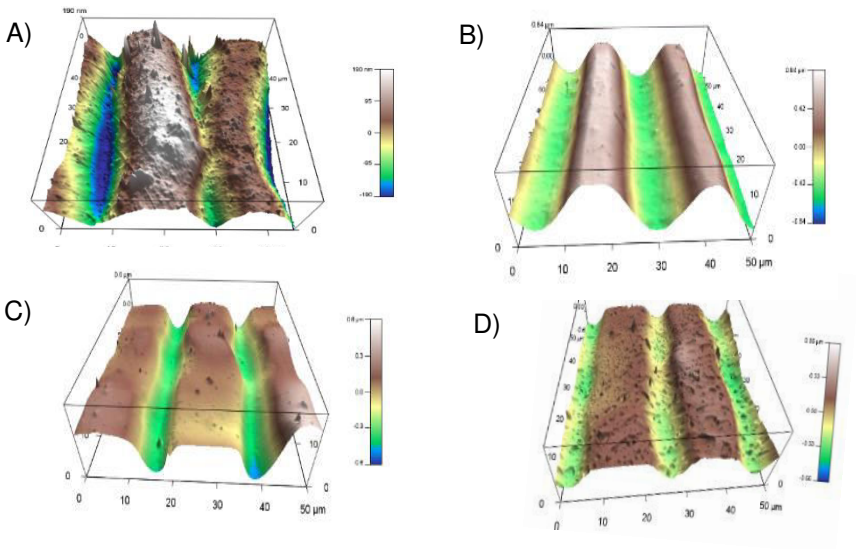

Figure 26: Topography of UV patterned gelatin. Atomic force microscopy 3D image of dry UV patterned samples resulting from A) unoptimized , B) optimized gelatin preparation using laser speed 80 mm/s. The effect of laser speed on patterning was tested comparing C) speed 90mm/s and D) speed 100 mm/s.

The trenches are not cut everywhere with the same depth; in some spots only 100 nm were achieved, while in other locations 500-1000 nm deep cuts were measured. If the cuts are deep, the excess gelatin is piled up on the side of the trenches or in the trenches, thereby blocking the interaction between cells. If the lines are not cut deep enough the cells lack a clear cue while forming tissue, thus leading to a poorly aligned cardiac tissue. Our hypothesis was that the laser encounters gas bubbles and debris on the gelatin which causes power fluctuations and variable patterning.Therefore, to improve the gelatin topography, we prepared cleaner, degased gelatin in order to get rid of the dust and gas bubbles. Also, the riboflavin

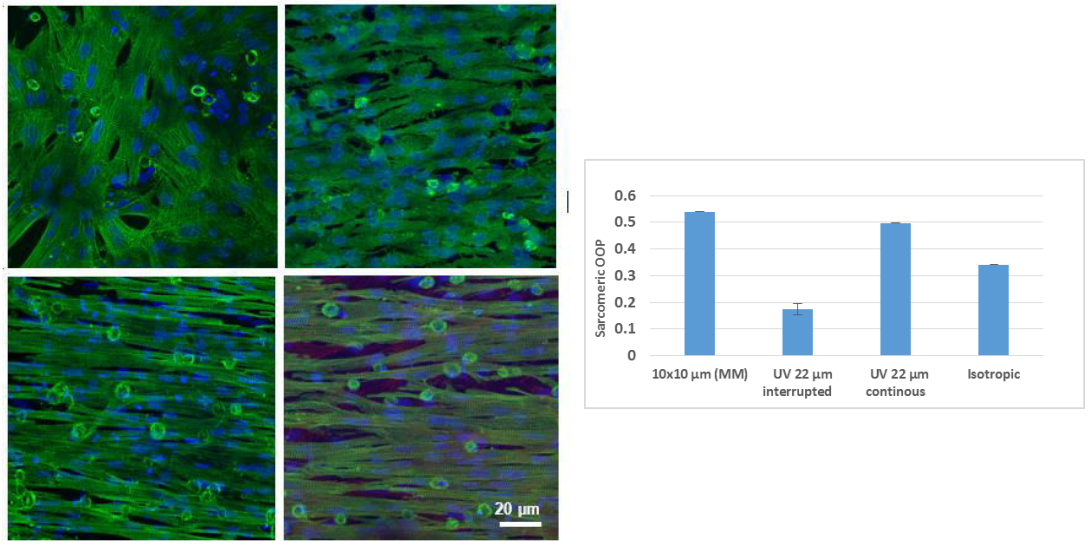

Figure 27: Quantitative sarcomeric alignment of engineered NRVM tissue. Confocal images of patterning different patterning techniques: A) isotropic, B) 10 µm x 10µm micromolded C) unoptimized UV patterned (speed 80, 22 µm), D) optimized UV patterning (speed 80, 22µm). DAPI stains for nuclei, α-actinin for sarcomeres. The graph shows, that the optimized UV patterned gelatin provides a surface for alignment almost as high as in case of micromolded sample. N=1, except for the UV 22 interrupted, where n=7 (not mosaic images), error bar is SEM.

was prepared in a higher concentration (0.3%) and the supernatant was filtered, thus reducing flakes. We tested the efficiency of laser speed 80, 90, 100 mm/s. Based on topography, with increasing speed the quality of gelatin pattering is decreased. The smoothest, best cut trenches could be achieved with laser speed 80 mm/s, whereas the 100 mm/s did not cut the surface of the gelatin.

The sarcomeres of NRVMs seeded on these particular samples could not be successfully immunostained therefore the alignment cannot be quantified by OOP Analysis. Comparing the alignment of actin and nuclei by eye, the best alignment appeared to be on the sample with speed 80. The optimization of image processing for OOP analysis was necessary, beside the local contrast and tubeness adjustment, the 6 confocal images of one sample are stitched together and the OOP software is run on the mosaic image. Using this analysis, the OOP of the isotropic tissue decreased from 0.41 to 0.34, which is the expected range.

After the optimization of gelatin preparation, laser speed and OOP analysis, NRVMs were seeded on gelatin with three different conditions (Figure 27). The sarcomeric alignment of 10x10 (10µm spaced by 10µm) micromolded lines is ~0.54. The alignment on UV patterned sample has OOP ~0.5, which is close enough to conclude that UV patterning might be suitable to achieve alignment as good as with micromolding.

4 Discussion

4.1 Development of functioning bi-layer heart-on-chip

4.1.1 Evaluating the flow setup for off-instrument culture of endothelial channel

By redesigning the manifold-based flow setup for endothelial cell culture in our microfluidic chip, we solved the previously encountered problems resulting from design flaws. The constant media perfusion of media has a great effect on the health of endothelial tissue. The heart-on-chip is not suitable for static culture, because of the gas- impermeable polycarbonate chip housing. One hour static incubation of freshly seeded HUVECs is found to be sufficient for the attachment of the cells to the ECM coated channel without causing any damage to the tissue. Endothelial cells are constantly exposed to shear stress resulting from blood flow and they are also able to transform mechanical stimulation into intracellular signalling events (Li, Haga, & Chien, 2005). The magnitude of shear stress in human venous system and in arteries was experimentally estimated for 1-6 dyn/cm^2 and 10-70 dyn/cm^2, respectively (Chiu & Chien, 2011). The magnitude of shear stress in mouse aorta is approximately one magnitude higher than in human (Suo et al., 2007). Higher than normal wall shear stress result in decreased endothelial barrier permeability (Buchanan, 2014), thus the pre-clinical mouse study cannot truthfully predict the magnitude of drugs transportation through the endothelium and the induced signalling pathways and the possible cardiotoxic effect. To match the flowrate that is used in other organs on chip for coupling, 60µl/hour flowrate was applied to both channel. Secondarily, the cardiomyocytes in the bottom of the chip do not support higher shear stresses, and applying different flow rate in the two channels would invalidate the standard barrier test which is necessary for drug testing. The applied 60 µl/hour flow rate cannot provide the normal the normal wall shear stress, but it is already beneficial to ECs (unpublished research of Kyung-Jin Jang). Computational studies suggested that the 60 µl/hour flowrate enables the drug added to the endothelial channel to reach the cardiomyocytes at relevant concentration, and the channel dimensions are a key parameter for setting final

drug concentrations. The height of the cardiac channel was reduced from 1.7 to 1.2 mm in order to achieve higher and more evenly distributed drug concentration. The manifold based setup can be used to culture the assembled chip if the interrogator is unavailable, and also is going to be used for culturing endothelial cells for the brain-on-chip and pancreas-on-chip, that are currently in development by our group.

4.1.2 Confluent endothelial tissue in heart-on-chip

The off-instrument flow set up was designed to allow not only for manual, but also for semi-automated seeding, that provides fast, efficient seeding and reduces the number of required cells. Since endothelial seeding, culturing and assembling the endothelial and cardiac parts are in an early developmental phase, the individual chips were seeded manually. We aim to reach confluent tissue within the shortest amount of time, therefore cells are seeded in high density. The best results were observed when 4-6 million cells/ml was seeded, cells seeded with 1 M cell / ml did not form a strong, confluent tissue. When ECs reach confluency they overgrow and cells start to delaminate from the surface. In order to avoid tissue damage due to its overgrowth, so once confluency is reached, the media is changed to one containing only 0.5% FBS, thereby reducing the proliferation activity of cell population. Currently the HUVECs tissue cultured in chip can be maintained for 2-3 weeks, but optimizing the ECM coating, e.g using a mixture of collagen and fibronectin might extends the lifetime up to at least 1 month.

4.1.3 Assembled chip on-instrument

The endothelial and cardiac chip have to be assembled with great caution as not to cause stress to the tissue and to avoid mistakes leading to microfluidic problems. The first cardiomyocyte MEA chip was damaged during the assembly, therefor no electrophysiological recordings could be done. The first assembled chip on interrogator experiment failed on day 1, because the connectors between cartridge and chip were too long, thus forming a seal with the bottom of both endothelial and cardiac channels and blocking the medium flow. In the next trials, further problems occurred, when the screws used to fix the chip to the cartridge were tightened slightly stronger on the cardiac channel, thus creating higher resistance, the flow is

going to escape to the top endothelial channel, thereby damaging the tissue barrier. The first indication of damaged to the endothelial barrier is the change in outflow media color, indicating inappropriate mixing between the channels. The cardiac media has a reddish color, while the endothelial media with FITC-inulin is light yellow-green, so any mixing is easily detected. Also, different amounts of outflow were collected from the endothelial and cardiac channels, indicating cross-membrane flow dues to differential channel resistance. Large differences in the resistance of the channels lead to invalidation of the barrier test, thus the extreme increase in leakage cannot be estimated. The same phenomenon was observed, when a bubble in endothelial channel endangered the health of the endothelial tissue underneath. The endothelial channel had to be pressurized to remove the bubble, thus damaging the endothelial barrier and causing media color change. The barrier test showed, that the leakage to the cardiac channel increased, so the endothelial cells had to recover from damage. The pressurizing event to remove bubbles takes about 10 minutes but influences the outcome of a 24 hours measurement.

The assembling also has to avoid bubble formation. When a bubble is formed, the source, location and size has to be identified. Not every bubble is harmful in the same way to the cells, depending on the diameter of bubble in vertical and horizontal direction, and some bubbles can be removed others cannot. Removing bubbles can lead to temporary tissue damage, but having a bubble constantly in the channel is lethal to the cells because it blocks the nutrients from underlying cells and decrease the channel diameter and hence increases the shear stress on the cells.

4.1.4 Verifying endothelial barrier function on interrogator

Altough the transportation of drugs through the endothelial layer happens selectively, we also need to measure a baseline diffusion that happens because of the leakage in between the cell-cell junction. This leakage also imeasures the quality of the engineered endothelial tissue. Therefore, we aimed to test the non-selective diffusion of FITC-inulin fluorescence tracer through the endothelial tissue cultured on the membrane. The addition of a HUVECs tissue on the top of the membrane decreased the leakage from ~38% to 5-10% with both the fibronectin and collagen

coated chips, but some differences could be observed. The fibronectin coated channel achieved the confluent tissue after one day in off-instrument culture, whereas the collagen coated cells needed one extra day to fully establish the cell-cell connections, as measured by barrier test. On day 15, endothelial medium started mixing with cardiac medium, thus the HUVEC layer cultured in collagen coated channel was considered damaged. In contrast, the fibronectin coated chips was kept in alive for 6 weeks with stable barrier function. To conclude that the fibronectin is prolonging the lifetime of HUVECs in culture, experiments with greater number of chips have to be carried out. The most promising ECM coating solution is the combination of the 100µg/ml collagen and 50µg/ml Fibronectin. Unpublished data by Ingber lab shows that HUVECs cultured in the lung-on-chip coated with a mixture of collagen and fibronectin can be in culture for up to one month. Maintaining viable endothelial tissue for four weeks would be beneficial, because the lifetime of cardiomyocytes cultured on gelatin is 4-6 weeks, thus longer term experiments could be carried out on the assembled heart-on-chip.

4.1.5 Cardiomyocytes in dual-channel heart-on-chip

Cardiac muscle cultured on MTFs in bi-layer heart-on-chip remained viable and functional during two-week on-instrument experiment. Further, the spontaneous beat frequency decreased and the contractile strength increased substantially. This indicates continuous maturation of the tissue, which can include cell thickening, more contractile fibers, and greater sarcomere spacing and thus a force increase. Beat rate reduction could not be observed in previously tested chip with no functional endothelial barrier, thus maturation might be an effect of chemical signals from the endothelial cells. To be able to prove tissue maturation and exclude the possibility of unhealthy cardiac tissue, pacing electrodes have to be inserted, so we are able to test the contractility when electrically stimulated.

4.2 Optical micropatterning for cardiac tissue engineering

Our group previously discovered that soft gelatin can be patterned by UV laser when riboflavin, a UV-sensitive crosslinking agent is used. Our aim was to test the

efficiency of this novel patterning method and compare the alignment of cardiac tissue to micromolding, which is currently the golden standard to pattern MTFs.

The first trial of seeding NRVMs on UV patterned gelatin samples had random alignment, therefore optimization was needed. We confirmed our hypothesis, that the discontinuous cell alignment was a result of improperly cut trenches and piled-up gelatin. A cleaner gelatin preparation and filtering of riboflavin resulted in a smooth gelatin surface and well-cut trenches. The sarcomeric alignment of isotropic tissue did not appear to be high when looking at stained images, but had an OOP 0.41. The software algorithm determined one primary axis of alignment and ignored the others, thus appeared to be a fairly well aligned tissue. By stitching the images into one mosaic image before the OOP analysis, the software recognized the previously determined primary alignments to be part of a bigger field of view, therefore taking the true angles and the OOP decreased to 0.34. The alignment of isotropic tissue has been reported as low as OOP 0.17 (McCain et al., 2014), but in that study the field of view was smaller, therefore inadequate for comparison. The orientation order parameter of discontinuous and continuous UV patterned tissue Figure 27 cannot be compared, because the images of the unoptimized condition was not stitched to a mosaic image, thereby leading to false OOP values. Assuming that stitching would improve the alignment of UV patterned tissue with two-fold, the OOP would be still in isotropic range.

Although the improvement of optimization cannot be truthfully quantified and higher sample numbers are needed, it can be concluded that the optimization had a positive effect on NRVMs alignment. Further, our result suggest that with UV patterning the orientation order parameter of micromolded tissue can be achieved, while improving accuracy, speed, and flexibility of patterning. Optical patterning can be a suitable method to replace micromolding in MTF fabrication for the bilayer heart-on chip.

5 Conclusion and future outlook

We developed optical patterning for soft hydrogels, that is faster, more accurate, operator independent, scalable and has a great potential in replacing the currently used manual molding in cardiac tissue engineering. Further adjustment and measurements are necessary, for example the quantification of patterned gelatin stiffness and the contractile strength of UV patterned MTFs. Also the method may be optimized for human cardiac iPS cells and can be applied for mimicking fibrosis by locally stiffening the gelatin.

With this study, we designed, built and tested an off-instrument flow setup for endothelial cell culture in chip, and established seeding and culturing protocol for HUVECs in a bi-layer heart-on-chip. Both endothelial and cardiac tissue remained viable and functioning during two-week on-instrument experiment, which validates the design of the bi-layer heart-on-chip. The daily endothelial barrier function test confirmed that the endothelial cells form a dense tissue within one day under flow condition, and barrier remains stable with minimal leakage to the cardiac channel for 6 weeks, depending on the extracellular matrix. At the same time, reduced spontaneous beat frequency and increased contractile strength of human iPS derived cardiomyocytes cultured on gelatin muscular thin films indicated the maturation of cardiac tissue. Further optimization is necessary to investigate the biochemical and signalling events occurring between the two layers in heart-on-chip. Currently, researchers are working on the replacement of the HUVECs with human cardiac microvasculature endothelial cell and also are integrating and testing electrical read out for endothelial confluency (TEER assay) in heart-on-chip. By implanting a microstimulator for electrical pacing in cardiac channel, we will be able to distinguish between unhealthy and mature cardiac tissue. Parallel development of custom MEAs are in

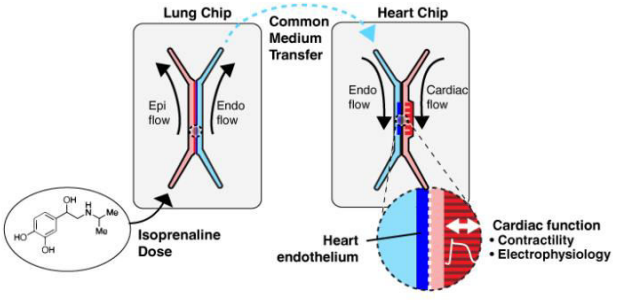

Figure 28: Schematic of drug study on coupled lung-and heart-on-chip. Isoprenaline is added to epithelial layer of lung-on-chip and transported via endothelial flow to the heart-on-chip. The effected endothelium triggers signalling events, that influences cardiac contractility and electrophysiology.

progress to be able to measure electrophysiological properties of cardiac tissue in chip. The finalization of key design features of heart-on-chip opens the path for studying biological responses. One could study the endothelium-mediated cardiotoxicity of cigarette smoke to confirm the hypothesis that the damage of cardiac vascular endothelium triggers inflammatory responses leading to myocardial pathology. Another potential research topic is to investigate the sexual dimorphism of drug induced long QT syndrome. Microfluidic coupling of two different organs-on-chips, e.g. heart and lung (Figure 28) can predict the cardiotoxicity of drugs (e.g. isoprenaline) that are used to treat pulmonary diseases. Further, by multi-organ chip coupling (e.g. heart, gut, and liver) more complex information on pro-drug responses and effect can be studied.

The sophisticated organs-on-chips platform has a great potential in pre-clinical drug development and also in the study of molecular mechanism of action and toxicity. This novel microfluidic *in vitro* model can help to identify new biomarkers of drug efficacy and toxicity, while modelling disease conditions. Great efforts are demonstrated in the linking of several organs-on-chip system to create a “human-on-chip” to accelerate the discovery of different avenues of drug delivery, as well as their efficacy and toxicity in humans, in a cheaper and ethical manner.
Although several studies successfully demonstrated organ level function on chip, several challenges need to be overcome, including technical robustness, like achieving optimal function of cells, fluidic control, bubble removal and gradient maintenance for periods over one month. Pharmaceutical industry relies on a high throughput screening approach that provides meaningful data for computational PK/PD models. To satisfy this requirement, in-line sensors of critical control and functional parameters (e.g. glucose, lactate, TEER, oxygen) has to be incorporated in chips. These next generation of chips ideally monitor overall system performance in real time (Bhatia & Ingber, 2014). The improvement of the technology would open up a broad range of application, like testing of dangerous agents (e.g. highly infectious virus) and isolating human cells from diseased groups or individual patient can accelerate the field of personalized medicine. Given the strict regulatory requirement and the complexity of the human body, it is unlikely that organ-on-chip can completely replace the animal studies in the near future, but the ad-

vantages and potential impact of organs-on-chip on drug development must be recognized by researcher and pharmaceutical industry.

List of References

Agarwal, A., Farouz, Y., Nesmith, A. P., Deravi, L. F., McCain, M. L., & Parker, K. K. (2013). Micropatterning Alginate Substrates for In Vitro Cardiovascular Muscle on a Chip. *Advanced Functional Materials*, *23*(30), 3738–3746. doi:10.1002/adfm.201203319

Agarwal, A., Goss, J. A., Cho, A., McCain, M. L., & Parker, K. K. (2013). Microfluidic heart on a chip for higher throughput pharmacological studies. *Lab on a Chip*, *13*(18), 3599–608. doi:10.1039/c3lc50350j

Bhatia, S. N., & Ingber, D. E. (2014). Microfluidic organs-on-chips. *Nature Publishing Group*, *32*(8), 760–772. doi:10.1038/nbt.2989

Chan, B. P., Hui, T. Y., Chan, O. C. M., So, K.-F., Lu, W., Cheung, K. M. C., … Yaroslavsky, A. (2007). Photochemical cross-linking for collagen-based scaffolds: a study on optical properties, mechanical properties, stability, and hematocompatibility. *Tissue Engineering*, *13*, 73–85. doi:10.1089/ten.2007.13.73

Chen, Y.-A., King, A. D., Shih, H.-C., Peng, C.-C., Wu, C.-Y., Liao, W.-H., & Tung, Y.-C. (2011). Generation of oxygen gradients in microfluidic devices for cell culture using spatially confined chemical reactions. *Lab on a Chip*. doi:10.1039/c1lc20325h

Chiu, J.-J., & Chien, S. (2011). Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. *Physiological Reviews*, *91*, 327–387. doi:10.1152/physrev.00047.2009

Dabiri, G. A., Turnacioglu, K. K., Sanger, J. M., & Sanger, J. W. (1997). Myofibrillogenesis visualized in living embryonic cardiomyocytes. *Proceedings of the National Academy of Sciences of the United States of America*, *94*, 9493–9498. doi:10.1073/pnas.94.17.9493

Davis, G. E., & Senger, D. R. (2005). Endothelial extracellular matrix: Biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. *Circulation Research*. doi:10.1161/01.RES.0000191547.64391.e3

Dlugosz, A. A., Antin, P. B., Nachmias, V. T., & Holtzer, H. (1984). The relationship between stress fiber-like structures and nascent myofibrils in cultured cardiac myocytes. *Journal of Cell Biology*, *99*, 2268–2278. doi:10.1083/jcb.99.6.2268

Dranka, B. P., Benavides, G. A., Diers, A. R., Giordano, S., Zelickson, B. R., Reily, C., … Darley-Usmar, V. M. (2011). Assessing bioenergetic function in response to oxidative stress by metabolic profiling. *Free Radical Biology & Medicine*, *51*, 1621–35. doi:10.1016/j.freeradbiomed.2011.08.005

Engler, A. J., Carag-Krieger, C., Johnson, C. P., Raab, M., Tang, H.-Y., Speicher, D. W., … Discher, D. E. (2008). Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. *Journal of Cell Science*, *121*, 3794–3802. doi:10.1242/jcs.029678

Feinberg, A. W., Alford, P. W., Jin, H., Ripplinger, C. M., Werdich, A. A., Sheehy, S. P., … Parker, K. K. (2012). Biomaterials Controlling the contractile strength of engineered cardiac muscle by hierarchal tissue architecture. *Biomaterials*, *33*(23), 5732–5741. doi:10.1016/j.biomaterials.2012.04.043

Feinberg, A. W., Feigel, A., Shevkoplyas, S. S., Sheehy, S., Whitesides, G. M., & Parker, K. K. (2007). Muscular thin films for building actuators and powering devices. *Science (New York, N.Y.)*, *317*, 1366–1370. doi:10.1126/science.1146885

Fujimoto, K. L., Ma, Z., Nelson, D. M., Hashizume, R., Guan, J., Tobita, K., & Wagner, W. R. (2009). Synthesis, characterization and therapeutic efficacy of a biodegradable, thermoresponsive hydrogel designed for application in chronic infarcted myocardium. *Biomaterials*, *30*, 4357–4368. doi:10.1016/j.biomaterials.2009.04.055

Gong, G., Liu, J., Liang, P., Guo, T., Hu, Q., Ochiai, K., … Zhang, J. (2003). Oxidative capacity in failing hearts. *American Journal of Physiology. Heart and Circulatory Physiology*, *285*, H541–H548. doi:10.1152/ajpheart.01142.2002

Gulino-debrac, D. (2013). Mechanotransduction at the basis of endothelial barrier function, (June), 1–10.

Houtkooper, R. H., Turkenburg, M., Poll-The, B. T., Karall, D., Pérez-Cerdá, C., Morrone, A., … Vaz, F. M. (2009). The enigmatic role of tafazzin in cardiolipin metabolism. *Biochimica et Biophysica Acta - Biomembranes*. doi:10.1016/j.bbamem.2009.07.009

Hsiao, A. Y., Torisawa, Y. suke, Tung, Y. C., Sud, S., Taichman, R. S., Pienta, K. J., & Takayama, S. (2009). Microfluidic system for formation of PC-3 prostate cancer co-culture spheroids. *Biomaterials*, *30*, 3020–3027. doi:10.1016/j.biomaterials.2009.02.047

Huh, D., Hamilton, G. A., & Ingber, D. E. (2011). From 3D cell culture to organs-on-chips. *Trends in Cell Biology*. doi:10.1016/j.tcb.2011.09.005

Huh, D., Leslie, D. C., Matthews, B. D., Fraser, J. P., Jurek, S., Hamilton, G. a, … Ingber, D. E. (2012). A human disease model of drug toxicity-induced pulmonary edema in a lung-on-a-chip microdevice. *Science Translational Medicine*, *4*(159), 159ra147. doi:10.1126/scitranslmed.3004249

Huh, D., Matthews, B. D., Mammoto, A., Montoya-Zavala, M., Hsin, H. Y., & Ingber, D. E. (2010). Reconstituting organ-level lung functions on a chip. *Science (New York, N.Y.)*, *328*(5986), 1662–8. doi:10.1126/science.1188302

Jacot, J. G., McCulloch, A. D., & Omens, J. H. (2008). Substrate stiffness affects the functional maturation of neonatal rat ventricular myocytes. *Biophysical Journal*, *95*, 3479–3487. doi:10.1529/biophysj.107.124545

Khalil, S., & Sun, W. (2009). Bioprinting endothelial cells with alginate for 3D tissue constructs. *Journal of Biomechanical Engineering*, *131*, 111002. doi:10.1115/1.3128729

Kim, S., Lee, H., Chung, M., & Jeon, N. L. (2013). Engineering of functional, perfusable 3D microvascular networks on a chip. *Lab on a Chip*, *13*, 1489–500. doi:10.1039/c3lc41320a

Li, Y. S., Haga, J. H., & Chien, S. (2005). Molecular basis of the effects of shear stress on vascular endothelial cells. *J Biomech*, *38*, 1949–1971. doi:S0021-9290(04)00458-0 [pii] 10.1016/j.jbiomech.2004.09.030

Marino, T. A., Kuseryk, L., & Lauva, I. K. (1987). Role of contraction in the structure and growth of neonatal rat cardiocytes. *The American Journal of Physiology*, *253*, H1391–H1399.

McCain, M. L., Agarwal, A., Nesmith, H. W., Nesmith, A. P., & Parker, K. K. (2014). Micromolded gelatin hydrogels for extended culture of engineered cardiac tissues. *Biomaterials*, *35*(21), 5462–71. doi:10.1016/j.biomaterials.2014.03.052

Meek, K. M., & Hayes, S. (2013). Corneal cross-linking - a review. *Ophthalmic and Physiological Optics*. doi:10.1111/opo.12032

Nguyen, P. D., Hsiao, S. T., Sivakumaran, P., Lim, S. Y., & Dilley, R. J. (2012). Enrichment of neonatal rat cardiomyocytes in primary culture facilitates long term maintenance of contractility in vitro. *AJP: Cell Physiology*. doi:10.1152/ajpcell.00449.2011

Olob, M. A. R. K. G., Oss, R. I. L. M., & Hesler, N. A. C. C. (2014). Cardiac Tissue Structure , Properties , and Performance : A Materials Science Perspective, *42*(10), 2003–2013. doi:10.1007/s10439-014-1071-z

Parker, K. K., & Ingber, D. E. (2007). Extracellular matrix , mechanotransduction and structural hierarchies in heart tissue engineering, (June), 1267–1279. doi:10.1098/rstb.2007.2114

Pedron, S., Van Lierop, S., Horstman, P., Penterman, R., Broer, D. J., & Peeters, E. (2011). Stimuli responsive delivery vehicles for cardiac microtissue transplantation. *Advanced Functional Materials*, *21*, 1624–1630. doi:10.1002/adfm.201002708

Rhee, D., Sanger, J. M., & Sanger, J. W. (1994). The premyofibril: evidence for its role in myofibrillogenesis. *Cell Motility and the Cytoskeleton*, *28*, 1–24. doi:10.1002/cm.970280102

Schaper, J., Meiser, E., & Stämmler, G. (1985). Ultrastructural morphometric analysis of myocardium from dogs, rats, hamsters, mice, and from human hearts. *Circulation Research*, *56*, 377–391. doi:10.1161/01.RES.56.3.377

Shinozawa, T., Imahashi, K., Sawada, H., Furukawa, H., & Takami, K. (2012). Determination of Appropriate Stage of Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes for Drug Screening and Pharmacological Evaluation In Vitro. *Journal of Biomolecular Screening*. doi:10.1177/1087057112449864

Smith, J. A., Shah, A. M., Fort, S., & Lewis, M. J. (1992). The influence of endocardial endothelium on myocardial contraction, *131*(March), 323–325.

Snibson, G. R. (2010). Collagen cross-linking: A new treatment paradigm in corneal disease - a review. *Clinical and Experimental Ophthalmology*. doi:10.1111/j.1442-9071.2010.02228.x

Sung, K. E., Yang, N., Pehlke, C., Keely, P. J., Eliceiri, K. W., Friedl, A., & Beebe, D. J. (2011). Transition to invasion in breast cancer: a microfluidic in vitro model enables examination of spatial and temporal effects. *Integrative Biology : Quantitative Biosciences from Nano to Macro*, *3*, 439–450. doi:10.1039/c0ib00063a

Suo, J., Ferrara, D. E., Sorescu, D., Guldberg, R. E., Taylor, W. R., & Giddens, D. P. (2007). Hemodynamic shear stresses in mouse aortas: Implications for atherogenesis. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *27*, 346–351. doi:10.1161/01.ATV.0000253492.45717.46

Wang, G., McCain, M. L., Yang, L., He, A., Pasqualini, F. S., Agarwal, A., … Pu, W. T. (2014). Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and heart-on-chip technologies. *Nature Medicine*, *20*(6), 616–23. doi:10.1038/nm.3545

Zervantonakis, I. K., Hughes-Alford, S. K., Charest, J. L., Condeelis, J. S., Gertler, F. B., & Kamm, R. D. (2012). Three-dimensional microfluidic model for tumor cell intravasation and endothelial barrier function. *Proceedings of the National Academy of Sciences*. doi:10.1073/pnas.1210182109