# COLLAGEN-GLYCOSAMINOGLYCAN SCAFFOLD SYSTEMS TO ASSESS HL-1 CARDIOMYOCYTE BEATING AND ALIGNMENT

BY

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THESIS

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## ABSTRACT

With heart disease being the leading cause of death in the US and an estimated 1.3 million heart attacks occurring annually, the need for tissue-engineered strategies to regenerate damaged cardiac tissue has become increasingly important. This thesis discusses the development of scaffold systems to examine the alignment and beating potential of HL-1 cardiomyoctyes in a 3D environment. Collagen-glycosaminoglycan scaffolds have been used extensively to probe the behavior of mature cells in vitro, but have not yet been designed for cardiac applications. In order to recapitulate key properties of the cardiac extracellular matrix, most notably its high degree of organization and alignment, we fabricated scaffolds with a longitudinally anisotropic pore structure. A freeze-dry process promoting unidirectional heat transfer through the precursor suspension was employed to create scaffolds of various mean pore sizes, all with pores elongated in the direction of solidification. The effects of structural cues on cell number, metabolic activity, alignment, and beating potential were quantified. It was shown that scaffolds with longitudinally anisotropic pore structures promoted spontaneous HL-1 cardiomyocyte beating compared to isotropic controls. This effect was dependent on pore size, with scaffolds with larger mean pore sizes exhibiting the highest instances of spontaneous beating. In addition, anisotropic scaffold variants promoted gross cell alignment in the longitudinal plane. These results indicate that an anisotropic collagen-glycosaminoglycan scaffold with larger pores (> 150 μm), may be most suited for cardiac tissue engineering applications.

## ACKNOWLEDGMENTS

This project would not have been possible without the support of many people. I want to start out by thanking my research adviser, Dr. Brendan Harley, for guiding me through my work. Brendan has been a wonderful mentor, and I am grateful that I had the opportunity to join his lab group. I would also like to thank the people who contributed directly to the work presented here. Lisa McGregor, an undergraduate research assistant in our lab, helped a great deal with the cardiomyocyte beating quantification experiments. I truly appreciate all her hard work on this project. Rory Turgeon and Dr. Ryan Bailey were key players in the photolithographic patterning of the CG scaffold array described in Appendix A. Thank you to my lab mates: Steven, Sunny, Bhushan, Dan, Jackie, Sara, Laura, Rebecca, Manny, Luke, Lisa, Doug and everyone else who has passed through the Harley lab during my time here – for making this lab a fun place to work, and for many helpful suggestions. Finally, thank you to my family, especially my mom and dad, who have always been there for me. And thank you to my husband, Matt: I love you and could not have done this without you.

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# CHAPTER 1: INTRODUCTION AND BACKGROUND

## 1.1 Cardiac Tissue Properties in Healthy and Diseased States

The heart is a muscle pump composed of connective tissue and various cell types. Cardiac fibroblasts are the most abundant cell type (70%) and are responsible for the production of type I and type III collagens that comprise much of the cardiac extracelluar matrix (ECM) (Weber 1989; Ohnishi, Sumiyoshi et al. 2007). Approximately one third of all cardiac cells are cardiomyocytes; however, cardiomyocytes comprise over two thirds of the myocardium, the muscular layer of the heart, by volume (Weber 1989). These cells are situated within a dynamic ECM environment that undergoes marked changes during cardiac tissue injury and disease.

### *1.1.1 The Cardiac Extracellular Matrix*

The most abundant components of the cardiac ECM are collagen, of which type I is the most prevalent (85%) followed by type III (11%), and proteoglycans (Bruggink, van Oosterhout et al. 2006). Heart valve leaflets contain a greater proportion (20%) of type III collagen (Weber 1989). In addition to being an integral part of the cardiac ECM, collagen is highly immunogenic and possesses functional groups that enable the binding of growth factors and other soluble molecules (Harley and Gibson 2008). Cardiomyocyte and collagen fiber orientation within the myocardium are highly organized and anisotropic in nature (Scollan, Holmes et al. 1998). The effective stiffness of the rat myocardium has been previously reported: $E_{\text{transverse}}$ ~54 kPa and $E_{\text{longitudinal}}$ ~20 kPa for the right ventricle; and $E_{\text{transverse}}$ ~157 kPa and $E_{\text{longitudinal}}$ ~84 kPa for the left ventricle (Engelmayr, Cheng et al. 2008).

*1.1.2 Cardiac Tissue Injury and Disease*

Cardiac tissue injuries, both acute and chronic, have a poor clinical outcome. Coronary heart disease is the leading cause of mortality in the United States, with an estimated 1.3 million heart attacks occurring each year (Lloyd-Jones, Adams et al. 2010). The inflammatory wound repair processes associated with cardiac tissue injury result in scar tissue formation, inducing ventricular structural changes and causing a loss of contractive function (Anversa, Kajstura et al. 2006). Myocardial remodeling refers to the structural changes that occur after a cardiac event or during cardiac diseased states to the ventricular wall architecture. The remodeling process is a combination of cellular and extracellular processes, and the cascade of events that occurs to create these structural changes is highly complex, involving numerous signaling pathways (Spinale 2007).

The infarcted myocardium is characterized by collagen fiber accumulation and increasing heart-chamber stiffness (Yamazaki, Shiojima et al. 1995; Fomovsky and Holmes 2010). Following myocardial infarction (MI), the wound repair process involves an inflammatory phase which overlaps with a tissue remodeling phase. Acute inflammation and tissue necrosis initially dominate this process, after which time chronic inflammation becomes increasingly important as fibroblasts aid in the production of connective tissue (primarily collagens) until the healing process is complete (Fishbein, Maclean et al. 1978). It should be noted that collagen degradation precedes this collagen accumulation process and resulting scar tissue formation (Judd and Wexler 1975). Matrix metalloproteinase (MMP-2, MMP-3, MMP-9) expression is induced in the ischemic regions of the heart, and expression of tissue inhibitors of matrix metalloproteinases (TIMP-1) are initially repressed following myocardial infarction (Romanic, Burns-Kurtis et al.

2001). The ECM reorganization associated with these processes contributes to cardiomycotye misalignment (Romanic, Burns-Kurtis et al. 2001). The resulting scarring from this imperfect healing process inhibits normal cardiac contractile function and can lead to congestive heart failure (Shah, Hung et al. 2011).

During left ventricular hypertrophy (ventricular enlargement resulting from either pressure or volume overload due to pathological changes associated with hypertension or other underlying disease states), the normally highly organized architecture of the myocardium is degraded by MMPs and reconstituted into an unorganized (and in the case of pressure overload hypertrophy, thickened) collagen network (Spinale 2007). Fibrosis results, in part due to an imbalance in MMP and TIMP levels, as well as the transition of cardiac fibroblasts to myofibroblasts (Berk, Fujiwara et al. 2007). Fibrosis disrupts synchronized cardiomyocyte contraction, a process facilitated by the myocardium ECM, during systole (contraction) and diastole (relaxation) (Berk, Fujiwara et al. 2007).

Congestive heart failure is characterized by an increase in markers of type I collagen synthesis (PICP and PINP) and a decrease in ECM degradation markers (MMP-1), which likely contribute to excess matrix deposition and fibrosis observed in patients with congestive heart failure (Alla, Kearney-Schwartz et al. 2006). Diabetes is also associated with the pathological heart and increased heart chamber stiffness (de Simone, Barac et al. 2008).

### 1.2 HL-1 Cardiomyocytes

HL-1 cells are a mouse-derived cardiomyocyte-like cell line that has been used extensively as a model system for adult cardiomyocytes. They have gene expression profiles similar to adult cardiomyocytes, including expression of α-cardiac myosin heavy chain, α-cardiac actin, and connexin43 (Claycomb, Lanson et al. 1998). They also express the cardiac markers desmin, sarcomeric myosin, and atrial natriuretic factor (Claycomb, Lanson et al. 1998). This cell line maintains a contractile cardiac phenotype out to at least passage 240, and the cells spontaneously beat in near-confluent culture (Claycomb, Lanson et al. 1998). This cell line has been used extensively as a model system for *in vitro* and *in vivo* studies of physiological and pathological cardiac conditions (Watanabe, Smith et al. 1998; White, Constantin et al. 2004; Mathur, Walley et al. 2011), as well as to explore the mechanisms regulating cardiomyocyte behavior (Hamacher-Brady, Brady et al. 2007; Salisch, Klar et al. 2011). In addition, this cell line is suitable for long-term *in vitro* studies (Smith, Segar et al. 2011).

### 1.3 Cardiac Tissue Engineering

Cells exist within a complex, three-dimensional structure that supports and regulates cell activity. With this understanding of the extracellular environment, scaffolds that serve as analogs of the native ECM have been created for a wide range of tissue engineering applications. Porous biomaterials been used *in vivo* as a regeneration template to induce a modification in the characteristic healing process following injury, as well as serve as standardized substrates for mechanistic studies of cell behaviors (migration, motility, contraction) *in vitro* (Freyman, Yannas et al. 2001; Harley, Kim et al. 2008). Scaffold microstructure (porosity, mean pore size, pore shape, interconnectivity, specific surface area) and mechanical properties (elastic modulus) have

been shown to significantly influence cell behaviors such as adhesion, growth, and differentiation *in vitro* and affect scaffold bioactivity *in vivo* (Harley and Gibson 2008). Cardiac tissue engineering is a field that aims to develop bio-compatible material structures that mimic key aspects of the myocardium for implantation *in vivo* and/or provide a 3D microenvironment in which to better understand the regulation of specific cardiac cellular mechanisms *in vitro*. These aspects may be structural, mechanical, chemical (or any combination thereof) in nature.

Researchers have attempted to recapitulate many aspects of the native myocardium, most notably its anisotropy. These efforts have focused predominately on 2D systems. Zong et al. created electrospun poly(L-lactide)-based (PLLA) mats with aligned macro-scale fiber orientations and found that primary cardiomyoctyes cultured on these constructs developed mature sarcomeres. (Zong, Bien et al. 2005). Orlova et al. determined that anisotropic electrospun polymethylglutarimide (PMGI) sheets promoted gross orientation of primary neonatal rat cell α-actin filiments (Orlova, Magome et al. 2011). Engelmayr et al. developed 3D poly(glycerol sebacate) scaffolds with accordion-like honeycomb shaped pores, and showed that the anisotropic structure guided neonatal rat heart cell alignment (Engelmayr, Cheng et al. 2008).

The effects of ECM mechanics on cardiomyoctye beating have also been explored in 2D and 3D substrates. Engler et al. found that embryonic cardiomyoctye beating was sensitive to matrix elasticity, with cells cultured on harder (e.g. scar-like stiffness) matrices slowing their beat frequency over days. In addition they determined that to maximize cardiac work, where matrix and cell strain are approximately equal, an optimal elastic modulus ($E^* \approx$ 11-17 kPa) exists

(Engler, Carag-Krieger et al. 2008). Shapira-Schweitzer and Seliktar found that neonatal rat cardiomyocytes cultured within compliant poly(ethylene glycol)ylated fibrinogen hydrogels demonstrated highly organized contraction patterns compared to those cultured within stiffer constructs (Shapira-Schweitzer and Seliktar 2007).

### 1.4 Collagen-Glycosaminoglycan Scaffolds

Collagen-glycosaminoglycan (CG) scaffolds are fabricated via freeze-drying (lyophilization) process from an acidic suspension of co-precipitated collagen and glycosaminoglycans. Glycosaminoglycans (GAGs) are long, unbranched polysaccharides that help make up proteoglycans. The copolymerization of type I collagen with GAGs serves to increase the fiber stiffness and decrease the degradation rate of the collagen (Harley and Gibson 2008). As the suspension is frozen, an interpentetrating network of ice crystals is formed around the co-precipitate; sublimation yields a highly porous scaffold (low relative density) with an interconnected pore structure. Control of freezing parameters, including thermal conductivity and freezing temperature, determines the growth rate of the ice crystals, allowing for the fabrication of scaffolds with distinct average pore sizes and shapes, including isotropic or anisotropic variants (O'Brien, Harley et al. 2004; Caliari and Harley 2011; Davidenko, Gibb et al. 2011).

CG scaffolds have been used for the regeneration of the skin, conjunctiva, peripheral nerve, and orthopedic tissue *in vivo* (Yannas, Lee et al. 1989; Harley, Spilker et al. 2004; Harley and Gibson 2008; Harley, Lynn et al. 2010). Cellular solids modeling has enabled an understanding of cells' local environments (Harley, Leung et al. 2007). The CG scaffold behaves like an elastomeric

foam in compression, exhibiting a linear elastic region, a collapse (buckling) plateau, and a densification region. (Harley, Leung et al. 2007). The elastic modulus of hydrated isotropic CG scaffolds is on the order of ~200 Pa; however with chemical (carbodiimide) cross-linking, this modulus can be increased roughly seven-fold (Harley, Leung et al. 2007).

### 1.5 Approach

To facilitate myocardial repair in a clinically relevant way, using a biomaterial, potentially combined with cell therapy, holds great promise. First we must understand how to design instructive tissue-engineered scaffolds in a way that promotes cardiac cell viability and proliferation, as well as maintenance of a cardiac phenotype. The CG scaffolds described previously have not yet been designed for cardiac applications. As cardiomyocytes are the cells responsible for reconstituting lost muscle tissue following injury, we decided to utilize these CG scaffolds to study the effects of microstructural cues on HL-1 cardiomyocyte behaviors. As the myocardium ECM is highly aligned, we fabricated scaffolds with isotropic or longitudinally anisotropic pores of differing sizes, to study the effects of anisotropy and pore size on HL-1 cardiomyocyte viability, alignment, and beating potential.

# CHAPTER 2: EXPERIMENTAL DESIGN

## 2.1. Collagen-GAG Scaffold Fabrication

CG scaffolds were fabricated via freeze-drying from a suspension of type I collagen from bovine tendon (Sigma Aldrich, St. Louis, MO) and chondroitin sulfate from shark cartilage (Sigma Aldrich, St. Louis, MO) in 0.05 M acetic acid. Scaffolds with axially aligned microstructures were fabricated using a directional solidification strategy that promotes unidirectional heat transfer during freezing (Caliari and Harley 2011). Briefly, the degassed CG suspension was pipetted into cylindrical wells (dia: 6 mm, height: 15 mm) in a polytetrafluoroethylene (PTFE) mold mounted on a 1/16" thick copper base. The mold was placed on a pre-cooled (-60, -40, -10ºC) freeze dryer shelf and held at the freezing temperature for 2 h to ensure complete solidification. The mismatch in thermal conductivity between the PTFE and copper ($k_{Cu}/k_{PTFE} \approx 1600$) induces directional solidification resulting in the formation of an anisotropic scaffold microstructure; decreasing freezing temperature results in scaffolds with decreasing pore size, but consistent degree of scaffold anisotropy (Caliari and Harley 2011).

Control (isotropic) scaffolds with isotropic microstructures were created using a constant cooling lyophilization method developed to create a uniform scaffold microstructure (O'Brien, Harley et al. 2004; O'Brien, Harley et al. 2005). Degassed suspension was poured into an aluminum tray and cooled at 1ºC/min until reaching a final freezing temperature of -40$^{o}$C. The suspension was held at the final freezing temperature for 1 h, resulting in an interpenetrating network of ice crystals surrounded by CG co-precipitate.

For all variants, after solidification ice crystals were sublimated under vacuum (0$^{o}$C, 200 mTorr), leaving behind scaffolds with an interconnected, porous microstructure. Scaffolds were sterilized and dehydrothermally cross-linked under vacuum (<25 Torr) at 105ºC for 24 h (Harley, Spilker et al. 2004). Individual scaffolds samples were cut into cylindrical plugs (dia: 6 mm, height: ~4 mm) using a biopsy punch (isotropic scaffold sheets) or razor blade (segments from cylindrical aligned scaffolds). Scaffolds were then immersed in 100% ethanol overnight, rinsed in phosphate-buffered saline without calcium and magnesium (PBS), and further cross-linked using carbodiimide chemistry in a solution of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDAC) and N-hydroxysulfosuccinimide (NHS) at a molar ratio of 5:2:1 EDAC:NHS:COOH (Olde Damink, Dijkstra et al. 1996; Harley, Leung et al. 2007). All scaffolds were rinsed in PBS and subsequently stored in PBS at 4ºC until use.

## 2.2 Cell Culture

### *2.2.1 Hl-1 Cell Culture*

HL-1 cells, a gift from Dr. William Claycomb, were maintained according to the protocols supplied by the Claybomb laboratory. Briefly, HL-1 cells were cultured on gelatin and fibronectin-coated tissue-culture flasks in Claycomb medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO), 100 U/mL:100 μg/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), and 0.1 mM norepinephrine (Sigma-Aldrich, St. Louis, MO). Cells were fed daily and cultured to confluence at 37$^{o}$C and 5% $CO_2$, after which they were split 1:3. Passage 99 HL-1 cells were used for scaffold experiments.

*2.2.2 Scaffold Culture Conditions*

Hydrated scaffold plugs were immersed in complete Claycomb medium (>1 h), blotted on sterile Kimwipes to remove excess liquid, and placed in low-attachment 6-well plates (Corning Life Sciences, Lowell, MA). HL-1 cells were seeded into the scaffolds using previously defined conditions (Harley, Kim et al. 2008; Caliari and Harley 2011). Briefly, HL-1 cells were trypsinized and resuspended at a concentration of 5 x $10^5$ cells/20 μL media. 10 μL of cell suspension was then pipetted onto each scaffold. The scaffold disks were incubated for 20 minutes to allow preliminary cell attachment, then flipped over and an additional 10 μL of cell suspension was added to each scaffold for a total of 5 x $10^5$ cells/scaffold. Cells were allowed to attach for 2 h, after which time complete medium was added to each well to completely submerge each scaffold. Scaffolds were cultured at 37ºC and 5% $CO_2$, and culture medium was exchanged daily.

**2.3 Determination of Cell Number**

The total number of cells per scaffold was determined using a DNA quantification assay (Kim, Sah et al. 1988; Caliari and Harley 2011). Scaffolds were placed in buffered papain solution at 60$^o$C for 24 h in order to digest the scaffolds and lyse the cells, exposing their DNA. Double-stranded DNA was fluorescently labeled with buffered Hoechst 33258 dye (Invitrogen, Carlsbad, CA) and assayed using a fluorometer (Tecan, Switzerland). Normalized fluorescent intensities were compared to a standard curve generated by measuring fluorescent intensities for known

numbers of cells. The total cell number within each scaffold was reported as a percentage of the initial seeding density.

### 2.4 Determination of Cell Metabolic Activity

The metabolic activity of the cells within each scaffold was determined using an alamarBlue assay (Tierney, Jaasma et al. 2009; Caliari and Harley 2011). AlamarBlue is a non-cytotoxic dye used as an indicator of cell health; it undergoes a colorimetric change when it is reduced by metabolically active cells. Cell-seeded scaffolds were rinsed in PBS then placed in a 10% solution of alamarBlue (Invitrogen, Carlsbad, CA) in complete culture medium and incubated under moderate shaking at 37$^{o}$C for 1 h. Changes in fluorescent intensity were measured using a fluorometer (Tecan, Switzerland), and normalized values were compared to a standard curve in order to determine an equivalent cell number for each scaffold. Relative metabolic activity per scaffold was reported as a percentage of the initial seeding density.

### 2.5 Determination of Cell Beating Fraction

The presence or absence of spontaneous HL-1 cell beating in an individual scaffold was determined by daily observation using a contrast-phase microscope (Leica Microsystems, Germany). A minimum of 6 distinct regions were examined per scaffold prior to designating the scaffold as ‘beating’ or ‘non-beating.’ Beating scaffolds were further classified as ‘center beating’ if the observed beating did not occur at the scaffold perimeter. Data was reported as the fraction of scaffolds of a particular variant designated as ‘beating’ or ‘center beating’ out of the total number of scaffolds of that variant examined on a given day.

### 2.6 Scaffold Histology

After 7 days in culture, a minimum of two scaffolds from each group were fixed in 10% neutral buffered formalin. The fixed scaffolds were embedded in paraffin wax and sectioned at 5 μm intervals along either the longitudinal (direction of directional solidification and scaffold anisotropy) or transverse (orthogonal to the direction of directional solidification and scaffold anisotropy) planes (one of each per group). Sections were stained for hematoxylin and eosin (H&E) to enable visualization of both the scaffold and cellular constituents (Caliari and Harley 2011).

### 2.7 Cell Orientation Analysis

Stained histological sections were visualized using a contrast phase microscope at 20x magnification. Images were captured for $n \geq 9$ distinct regions per variant for a total of at least 150 cells per plane per variant. Histology images were then imported into ImageJ. Cells were identified using the ellipse tool in ImageJ, and the orientation angle of each cell [$-90^{o} \leq x \leq +90^{o}$] was calculated using the 'measure feature' of the ImageJ add-on OrientationJ. Images were captured and analyzed in a manner such that an orientation angle of $0^{o}$ indicated cell alignment in the direction of directional solidification and pore anisotropy, while an orientation angle of +/- $90^{o}$ indicates cell alignment in the orthogonal direction (Caliari and Harley 2011).

### 2.8 Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test was performed on cell metabolic activity and cell number data. For cell alignment analysis, orientation angles

were grouped into $20^{o}$ histogram bins and results compared between bins; significant differences between variants were determined using Fisher's Exact Test. For cell beating experiments, differences in beating potential between scaffold variants were determined using Fisher's Exact Test; this was done for differences between groups at specific time points, as well as for time-collapsed data. Significance level was set at $p < 0.05$. At least $n = 6$ samples were examined at each time point for cell metabolic activity and cell number assays, and $n = 10$ samples were examined for beating analysis at each time point for up to 7 days in culture. After this point in time, the number of samples examined per group was $\geq 4$.

# CHAPTER 3: RESULTS

## 3.1 Scaffold Pore Structure Analysis

The average pore size and shape of these scaffolds have been previously characterized using stereological methods (Caliari and Harley 2011). Both the transverse and longitudinal planes of the scaffolds were analyzed to determine the degree of pore elongation (aspect ratio). The pre-cooling thermal treatments of -60, -40, and -10ºC resulted in scaffolds with elongated pores with average pore sizes of 55 ± 18 μm (transverse A.R. 1.07 ± 0.04, longitudinal A.R. 1.41 ± 0.16), 152 ± 25 μm (transverse A.R. 1.17 ± 0.08, longitudinal A.R. 1.67 ± 0.17), and 243 ± 29 μm (transverse A.R. 1.19 ± 0.12, longitudinal A.R. 1.57 ± 0.23), respectively (Caliari and Harley 2011). The isotropic control had an isotropic microstructure (A.R. 1.05 ± 0.03), with an average pore size of 87 ±10 μm (Caliari and Harley 2011; Martin, Caliari et al. 2011). To simplify matters, the aligned scaffolds will hereforth be referred to by their average pore size (‘55 μm’, ‘152 μm’, ‘243 μm’), while the control will be referred to as ‘isotropic.’

## 3.2 Scaffold Cell Number and Metabolic Activity

Both the aligned and isotropic scaffold variants supported metabolically active cells out to 14 days in culture (**Figure 3.1**). No significant differences between groups were observed in initial attachment as determined by cell DNA quantification at day 1 (**Figure 3.2**). This result was not unexpected, as all scaffolds received the same cross-linking treatment (dehydrothermal cross-linking followed by EDAC chemical cross-linking). We have observed previously that an increased degree of chemical cross-linking correlates to higher initial attachment rates for scaffolds with the same microstructure (aspect ratio, pore size, data not shown). This is likely

due to the static cell-seeding method employed, as cross-linked scaffolds are more easily dried prior to the addition of cell solution. While the number of cells supported on each scaffold decreased slightly from day 7 to day 14; over the same time period, the metabolic activity of the cells (or metabolic activity per scaffold) increased. Significant differences ($p < 0.05$) in cell number were observed at day 7 between the 55 μm variant and the isotropic control. Significant differences ($p < 0.05$) in cell metabolic activity were observed at day 7 between the 152 μm variant and the isotropic control.

### 3.3 HL-1 Beating in 3D Scaffold Culture

As described previously, cell-laden scaffolds were designated one of three ways: 'beating', which included a sub-classification of 'center beating', or 'non-beating'. The emphasis on beating location was due to the following reasoning: First, to ensure that the scaffold could support viable, beating cardiomyocytes at all radial locations in the scaffold despite diffusional limitations that may exist; and second, to ensure that the cells were in fact responding to microstructural cues, as cells located on the scaffold perimeter could be responding exclusively to cell-cell contacts. For statistical analysis, 'beating' proportions were compared to 'non-beating' proportions, and 'center-beating' proportions were compared to all non-'center beating' proportions of the total events examined using Fisher's Exact Test.

'Beating' fraction for each day in culture is shown in **Figure 3.3**, and a more simplified version of this data for days corresponding to when cell number and metabolic activity were quantified is

shown in **Figure 3.4**. A significantly higher proportion of the scaffolds were classified as 'beating' (as opposed to 'non-beating') after 1 day in culture for the following variants: ; 152 μm variant vs. isotropic control ($p = 0.05$); 243 μm variant vs. isotropic control ($p = 0.05$). Furthermore, a significantly higher proportion of the 243 μm variant was classified as 'center beating' compared to the isotropic control after 2, 3, and 4 ($p = 0.05$) days in culture (**Figure 3.5**). A simplified representation of 'center beating' fraction corresponding to days where cell number and metabolic activity were quantified is shown in **Figure 3.6**.

Collapsing across time, additional statistically significant differences for 'beating' vs. 'non-beating' classifications were observed between the different scaffold variants (listed as higher 'beating' proportion vs. lower 'beating' proportion): 152 μm variant vs. isotropic control ($p = 0.0002$); 243 μm variant vs. isotropic control ($p < 0.0001$); 152 μm variant vs. 55 μm variant ($p = 0.0016$); and 243 μm variant vs. 55 μm variant ($p = 0.0006$). For a time-collapsed comparison of 'center-beating' vs. non-'center-beating' classifications, the following scaffold variants exhibited significant differences (listed as higher proportion vs. lower proportion classified as 'center-beating'): 55 μm variant vs. isotropic control ($p = 0.0378$); 152 μm variant vs. isotropic control ($p < 0.0001$); 243 μm variant vs. isotropic control ($p < 0.0001$); 243 μm variant vs. 55 μm variant ($p = 0.0001$); and 243 μm variant vs. 152 μm variant ($p = 0.026$).

Our results indicate that an anisotropic scaffold microstructure more effectively promotes HL-1 cardiomyocyte beating in CG scaffolds. In addition, this effect is dependent on scaffold pore

size: We consistently observed higher beating fractions in scaffolds in the 152 μm and 243 μm pore size variants, with center-localized beating occurring most frequently in the 243 μm variant. We did not observe any center-localized beating in the isotropic control at days 1, 4, 7, or 14, where there were no significant differences in cell number or metabolic activity (with the exception of one aligned variant at day 7 for each case).

### 3.4 HL-1 Cell Alignment in Scaffold Variants

The anisotropic CG scaffolds used in this study have been previously shown to promote equine tendon cell alignment in the longitudinal plane. As the native myocardium is anisotropic, we expected to observe this same phenomenon in HL-1 cardiomyocytes. Representative scaffold cross-sections showing the pore elongation and cell alignment trends in the longitudinal plane and random cell orientation in the transverse plane after 7 days in culture can be found in **Figure 3.7**.

For a completely random cell orientation, as one would expect to see in an isotropic microstructure, we would anticipate an orientation angle distribution represented as a straight line at 5.5%, the percentage that would indicate each 10º bin from (-90º, -80º) to (+80º, +90º) being represented equally. The orientation angle distribution for the isotropic and all anisotropic scaffold variants in the transverse plane appears random, hovering around that 5.5% mark, and we observed no clear trend of alignment in any given direction (**Figure 3.8**).

Comparing cell orientation angles in the scaffold variants' longitudinal plane is much more interesting, as clear differences emerge between the isotropic control and the aligned scaffold variants (**Figure 3.9**). Compared to the isotropic control, a greater proportion of the cells had orientation angles that fell in the (-10º, +10º) range, a range considered to be in the general direction of pore alignment/elongation, for the 55 μm variant ($p = 0.0028$), 152 μm variant ($p = 0.0001$), and 243 μm variant ($p = 0.002$). Furthermore, these same variants were less likely to have cell orientation angles fall in the ranges of (-90º, -70º) and (+70º, +90º), a range that can generally be considered perpendicular to the direction of scaffold pore alignment, when compared to the isotropic control. The differences in proportions for the above-mentioned orientation angle ranges between each aligned variant and the isotropic control were statistically significant, each with a p value of $<0.0001$.

We also looked to examine the effect of pore size on cell alignment in the longitudinal plane. Though it appears that the anisotropic scaffold with the smallest pore size promoted increased cell alignment compared to the anisotropic scaffold with the largest pore size, i.e. a higher proportion of the orientation angles fell in the (-10º, +10º) bin for the 55 μm variant compared to the 243 μm variant, this difference was not large enough to be statistically significant ($p = 0.1647$). Expanding the range of orientation angles examined from (-10º, +10º) to (-20º, +20º), made this difference more prominent ($p = 0.1496$), but still not statistically significant. Comparing the proportion of cell orientation angles that fell in the (-10º, +10º) range for the 55 μm variant compared to the 152 μm variant was not statistically significant ($p = 0.6142$), but this

difference became much more pronounced when the range was expanded to (-20º, +20º) ($p = 0.0613$).

### 3.5 Figures

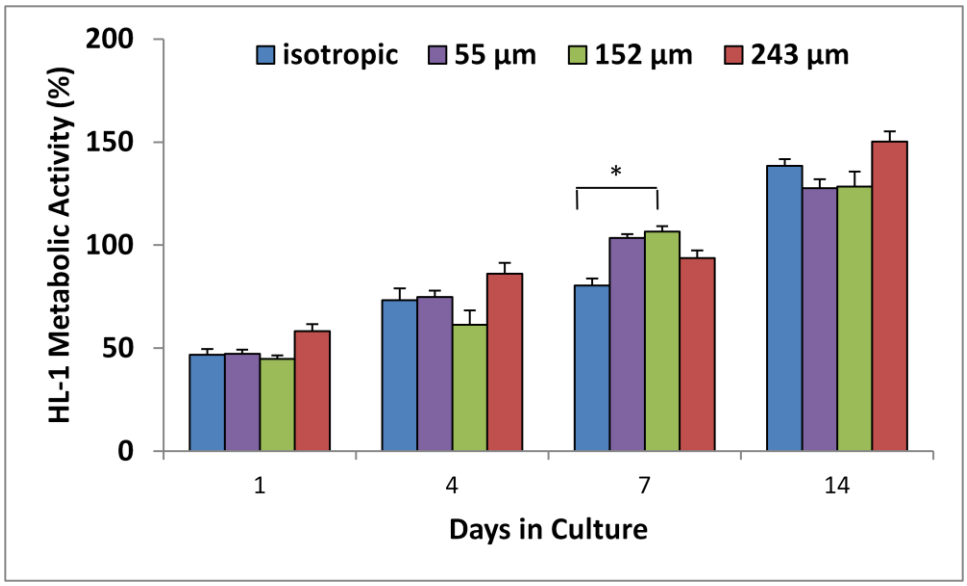



**Figure 3.1**. HL-1 equivalent cell number expressed as a percentage of the initial seeding density. Cell number was determined via a colorimetric alamarBlue assay. Significant differences ($p<0.05$) are indicated with an asterisk. Error bars are ±SEM.

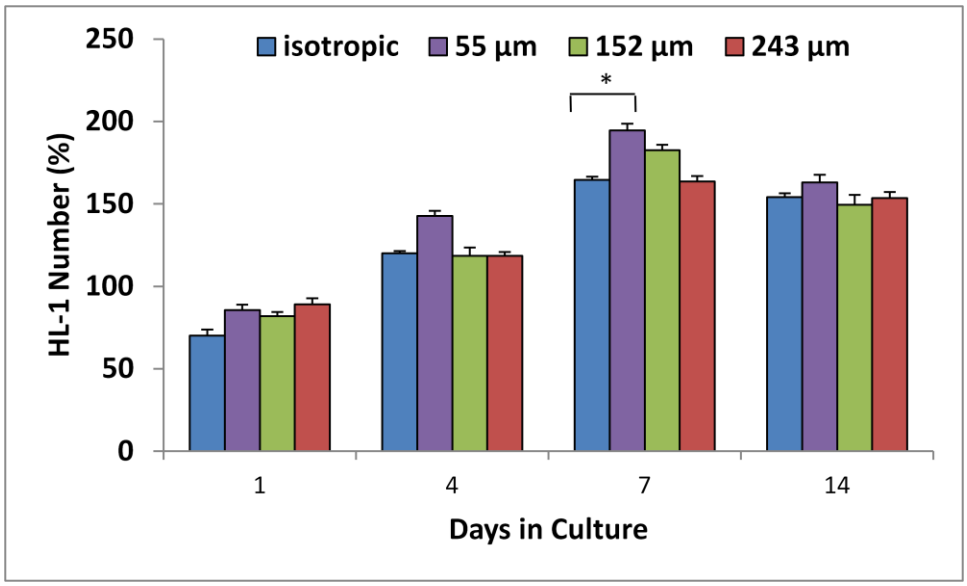



**Figure 3.2**. HL-1 cell number expressed as a percentage of the initial seeding density. Cell number was determined via DNA quantification using a Hoechst dye. Significant differences ($p<0.05$) are indicated with an asterisk. Error bars are ±SEM.

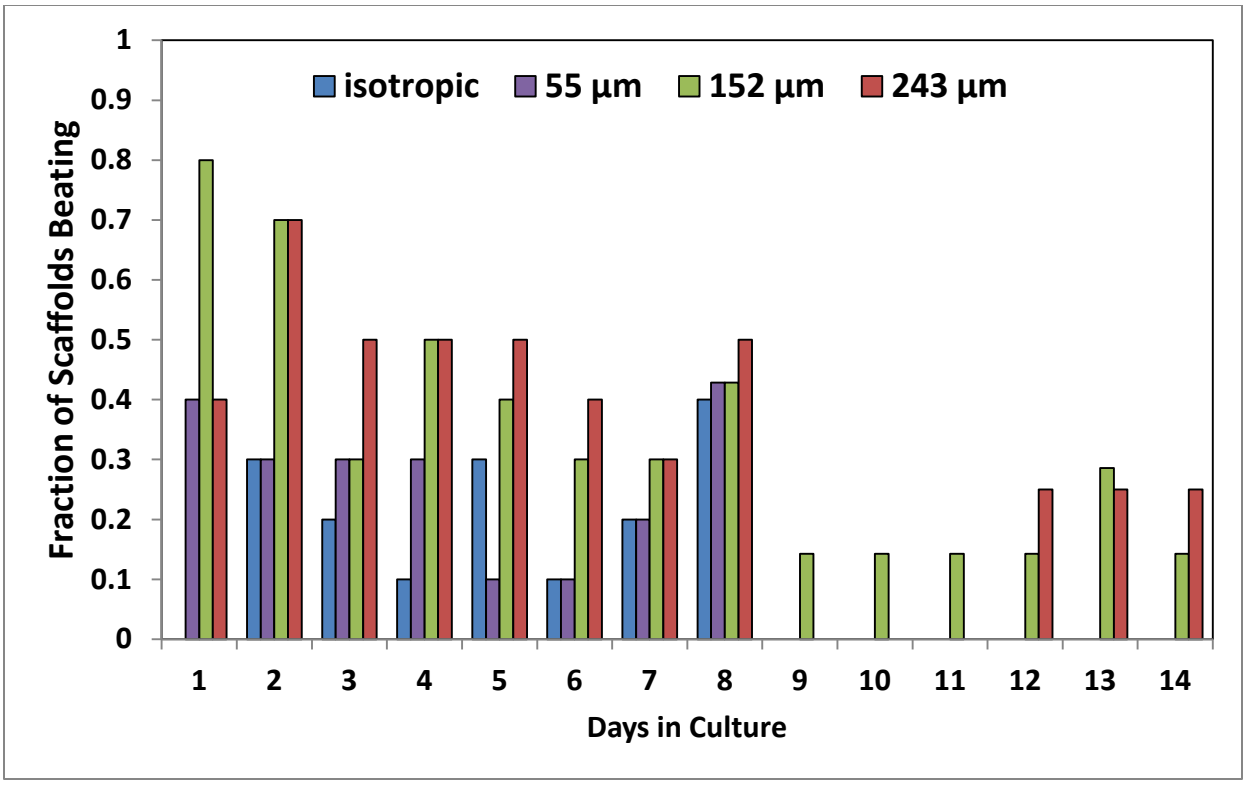



**Figure 3.3.** Fraction of scaffolds classified as 'beating' out of all scaffolds observed via contrast phase microscope visualization. Note that this fraction includes scaffolds classified as 'center beating' as well as scaffolds that exhibited beating near the scaffold edge.

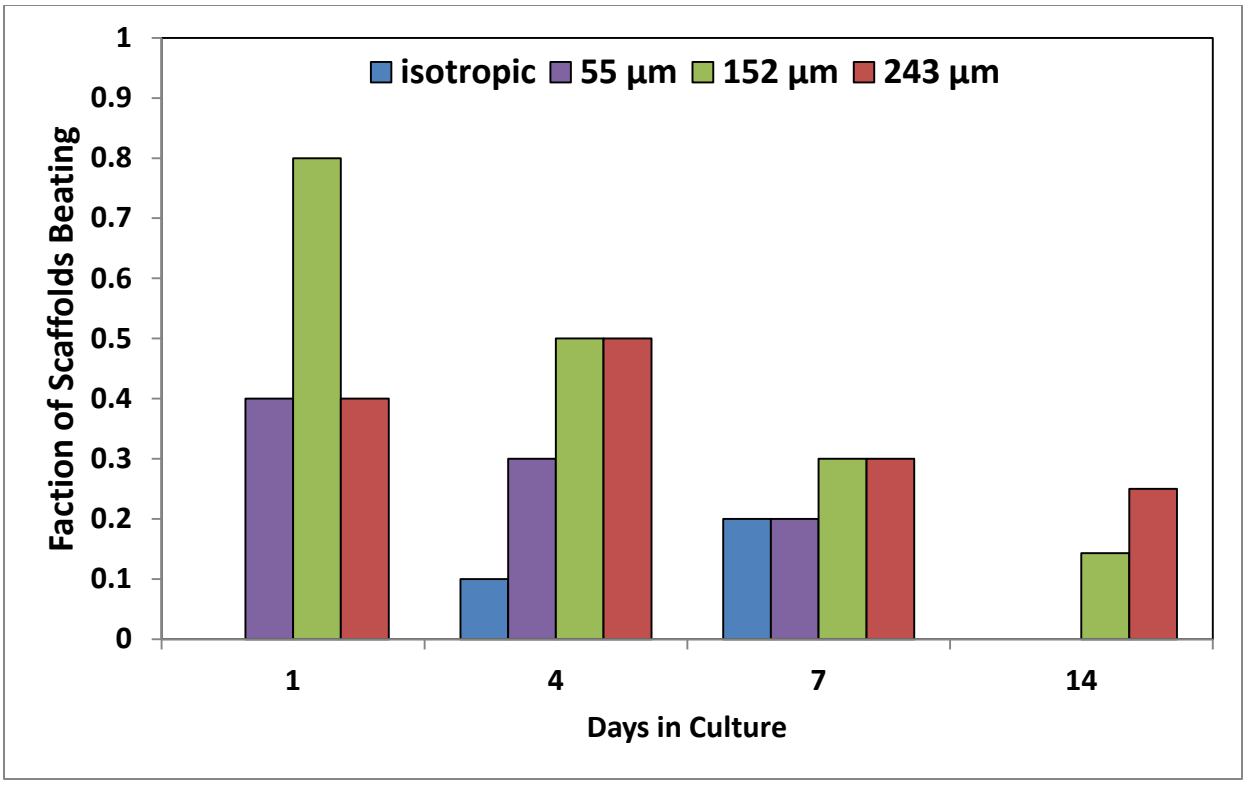



**Figure 3.4.** Fraction of scaffolds classified as 'beating' out of all scaffolds observed via contrast phase microscope visualization. Days shown correspond to days selected for metabolic activity and DNA quantification analysis. Note that this fraction includes scaffolds classified as 'center beating' as well as scaffolds that exhibited beating near the scaffold perimeter.

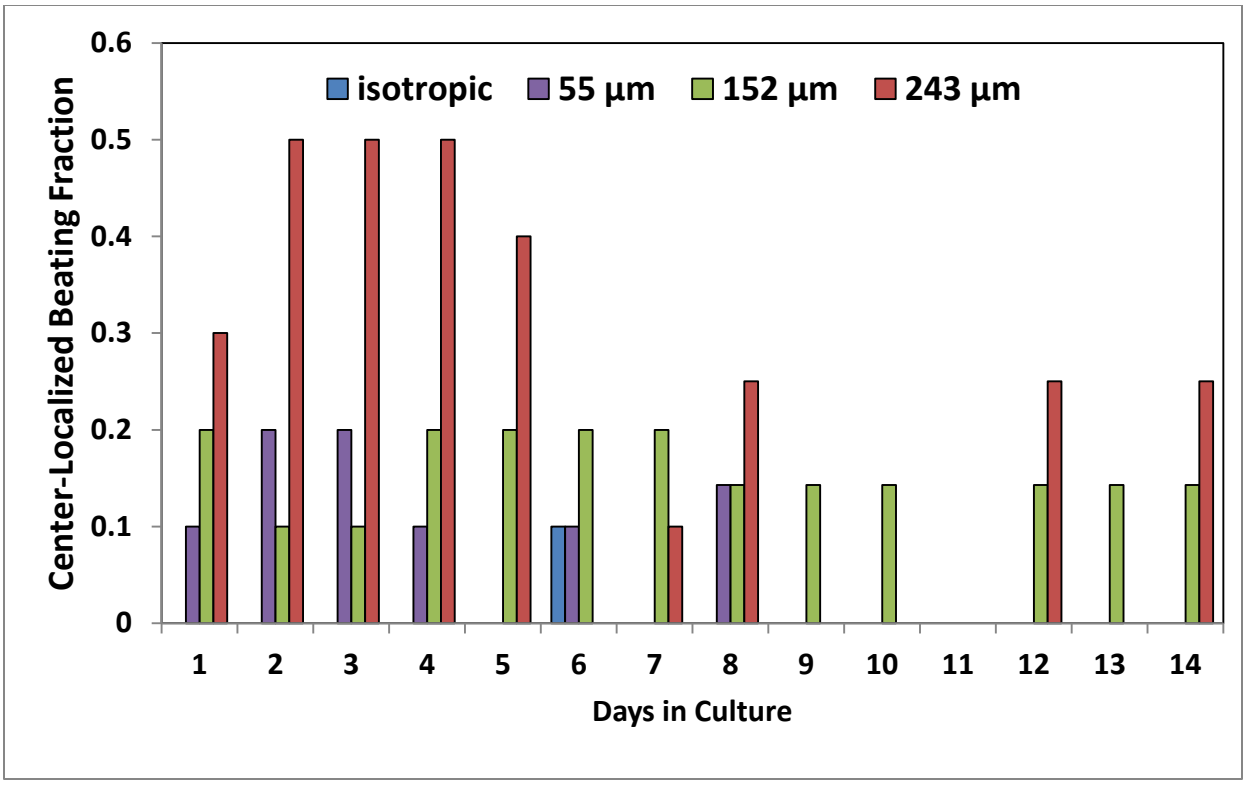



**Figure 3.5.** Fraction of scaffolds classified as 'center beating' out of all scaffolds observed via contrast phase microscope visualization.

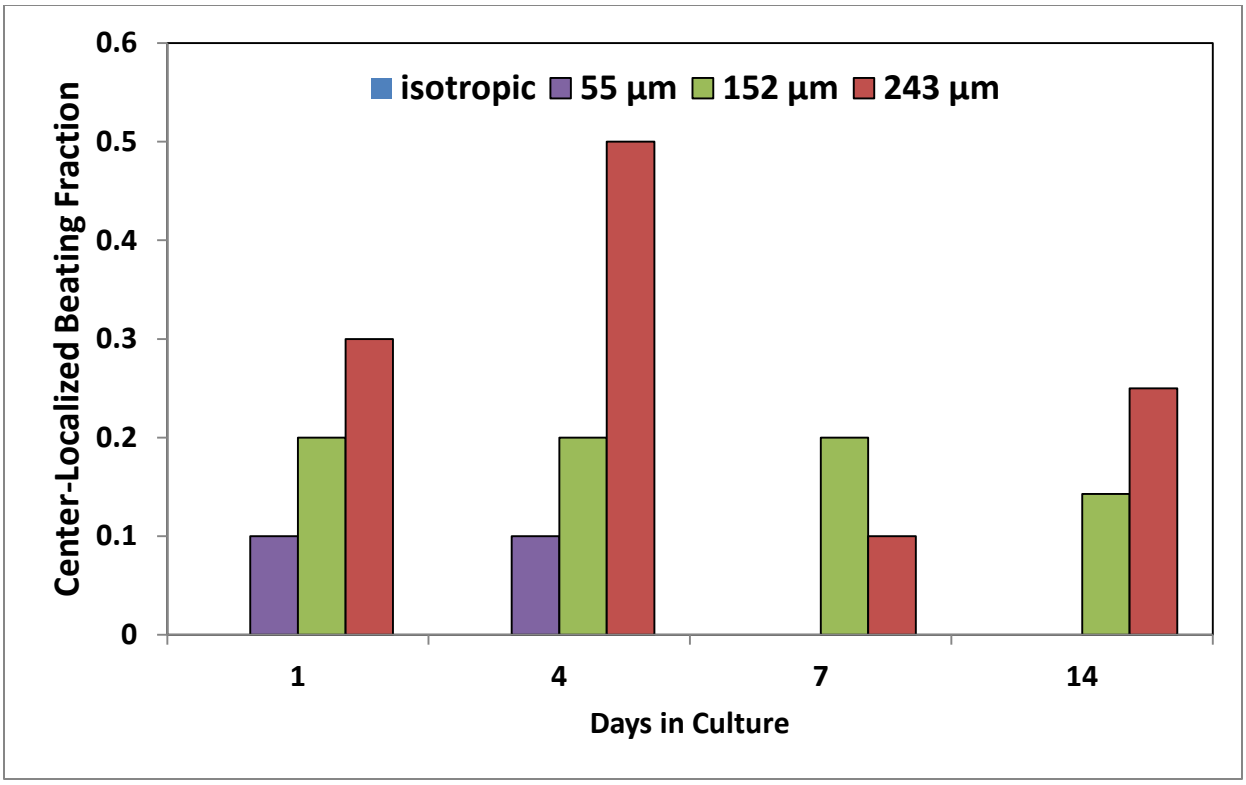



**Figure 3.6.** Fraction of scaffolds classified as 'center beating' out of all scaffolds observed via contrast phase microscope visualization. Days shown correspond to days selected for metabolic activity and DNA quantification analysis.

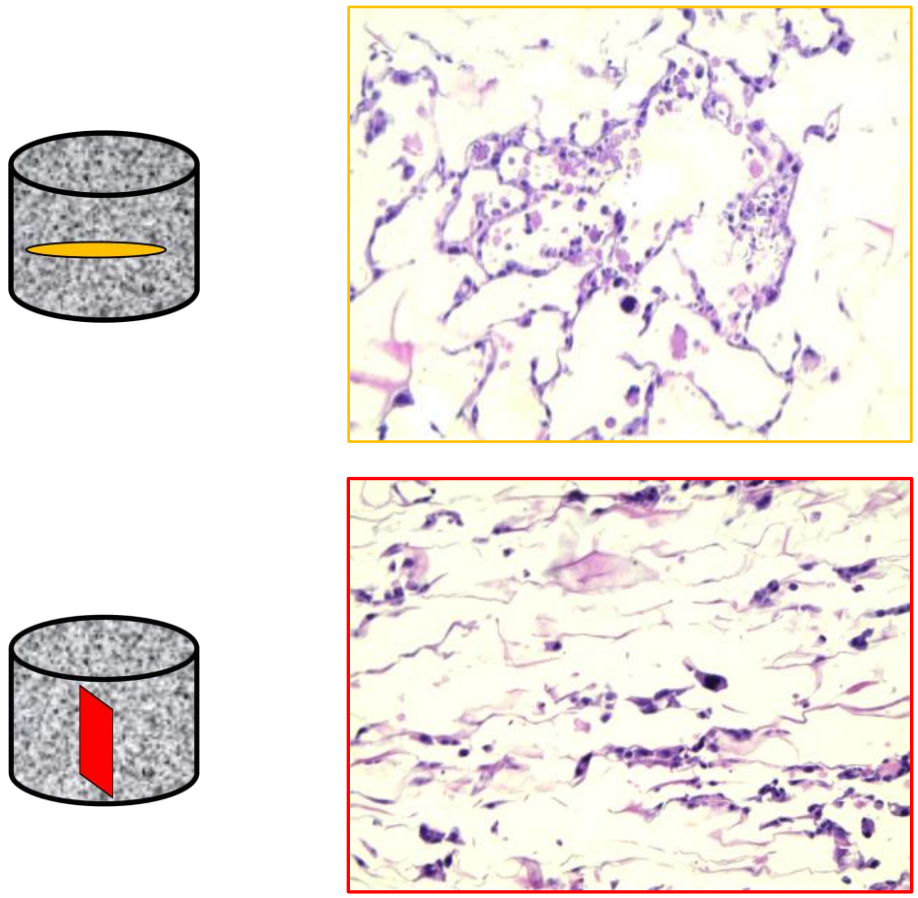


**Figure 3.7**. Representative transverse (yellow border) and longitudinal (red border) histological cross-sections for an anisotropic scaffold variant used to calculate cell orientation angles of cells (purple) situated within the scaffold pore structure (pink). Images were captured at 20x magnification on a contrast-phase microscope.

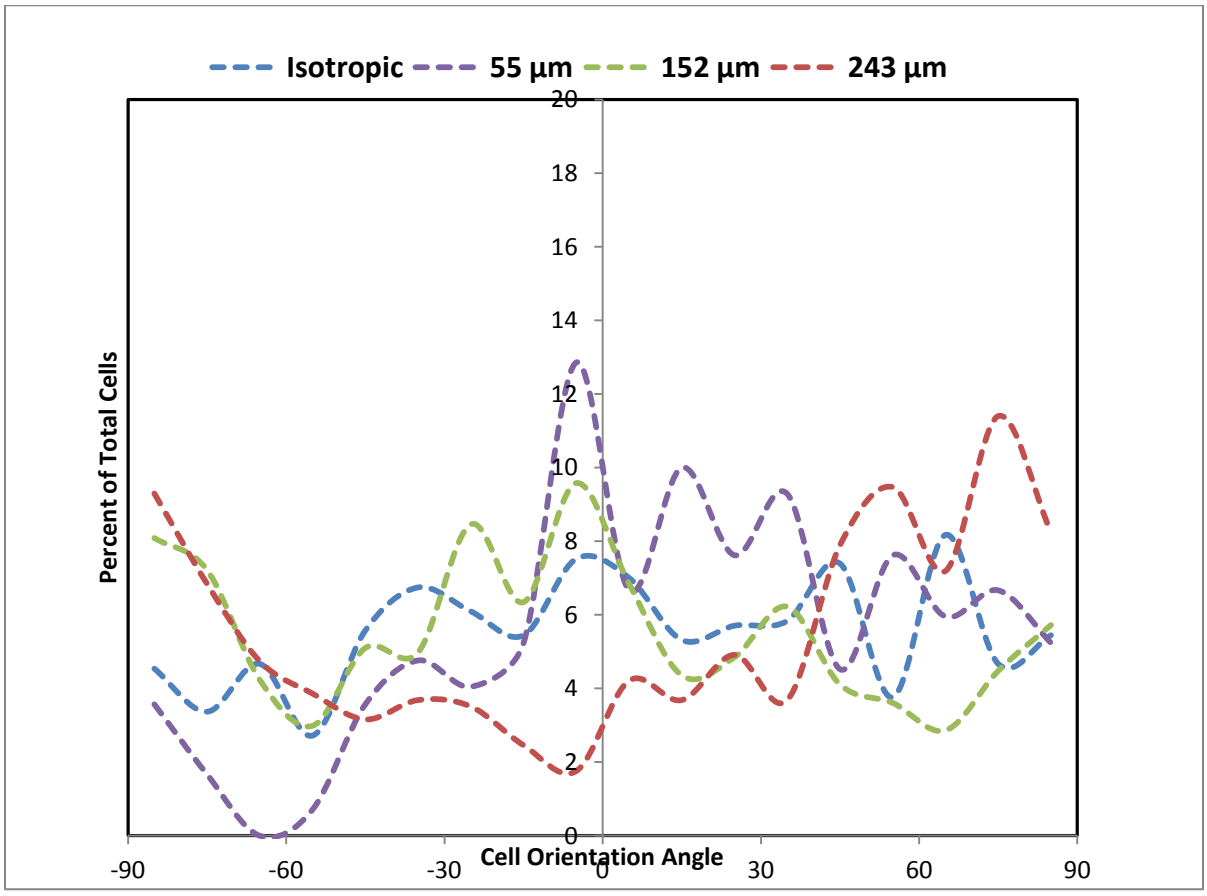



**Figure 3.8.** Orientation angle analysis of cells situated in all scaffold variants for representative transverse cross-sections after 7 days in culture. The horizontal axis indicates orientation angle, grouped into 10º bins, where an orientation angle of 0º indicates orientation in the direction of pore alignment, and ± 90º indicates orientation perpendicular to pore alignment. The vertical axis indicates the percentage of all cells examined for a particular variant that fell within the 10º orientation angle bins.

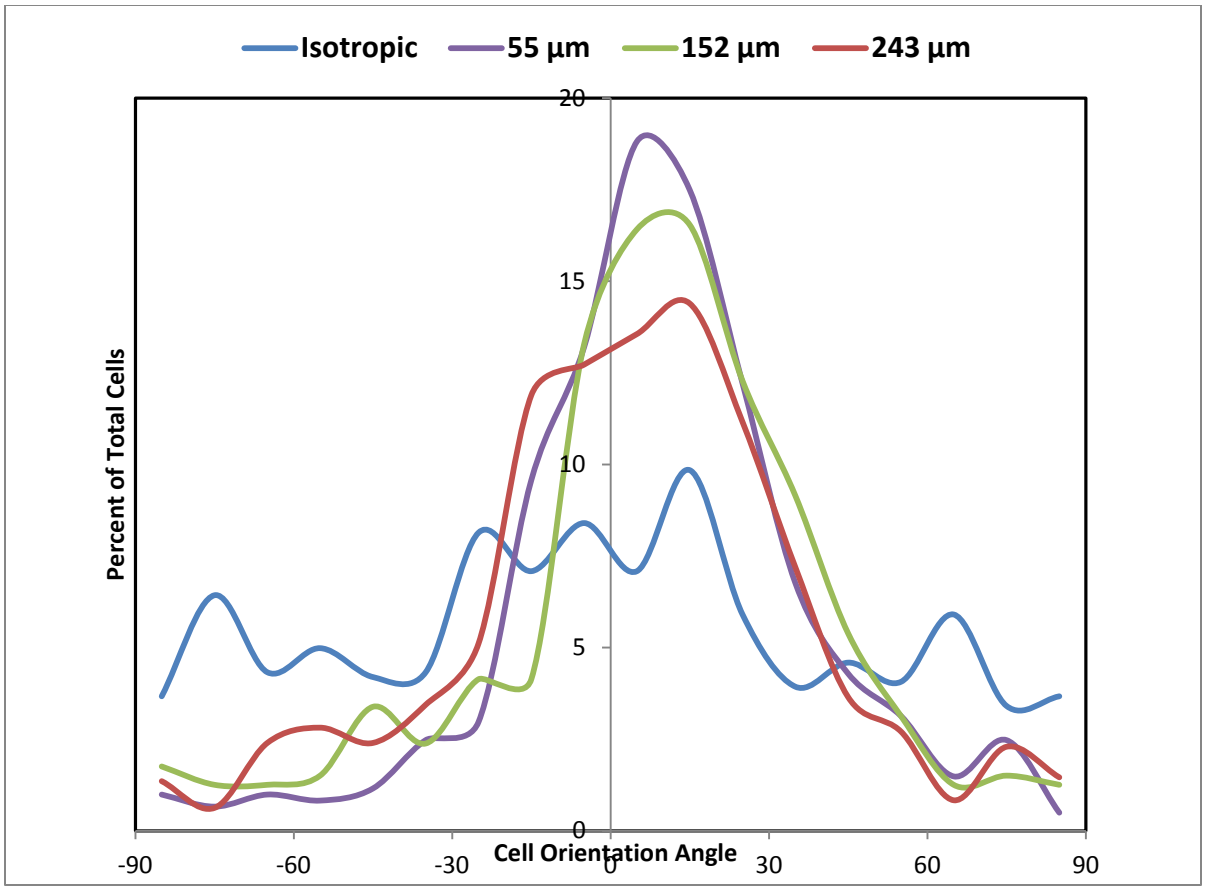



**Figure 3.9.** Orientation angle analysis of cells situated in all scaffold variants for representative longitudinal cross-sections after 7 days in culture. The horizontal axis indicates orientation angle, grouped into 10º bins, where an orientation angle of 0º indicates orientation in the direction of pore alignment, and ± 90º indicates orientation perpendicular to pore alignemtn. The vertical axis indicates the percentage of all cells examined for a particular variant that fell within the 10º orientation angle bins.

# CHAPTER 4: COLLAGEN-GAG SCAFFOLD ARRAYS

## 4.1 Background and Motivation

No tool currently exists to explore 3D microenvironmental cues on cells in a combinatorial and high-throughput manner. We have developed the ability to create a homologous series of microenvironments using CG scaffolds, "CG scaffold arrays." The array consists of a single chip mounted on a removable base. By varying local thermal conductivity during freezing, scaffold pore structure can be varied in one direction; biomolecule immobilization can be varied in the other (**Figure 4.1**). The chip then can continue on through cell culture and imaging steps, serving as a pseudo-well plate (the spacing and geometry of the nodes is designed to be compatible with existing well plate reader technologies).

## 4.2 Array Fabrication and Structural Analysis

Scaffold arrays with two distinct microstructural regions were fabricated by controlling local heat transfer during the freeze-dry process. A specialized mold was created for this purpose, consisting of a polysulfone chip (2 mm thick) with circular holes (6.5 mm diameter) drilled all the way through it, mounted on a removable base. The geometry/spacing of the nodes was designed to be identical to the dimensions of a 96-well plate. The removable base contained an aluminum section and a polysulfone section ($k_{aluminum}/k_{polysulfone} \approx 850$); this disparity in thermal conductivity was intended to control local heat transfer, and subsequently ice crystal growth kinetics, during freezing. An acidic suspension of 0.5% type I collagen (Sigma/matrix) and chondroitin sulfate from shark cartilage was pipetted into the array mold, and was frozen at a rate of 1ºC/min to a final freezing temperature of -10ºC or -40ºC. The freeze-dryer shelf was then held at the final freezing temperature for 2 h to ensure complete solidification prior to

sublimating out the ice crystals. After freeze-drying, the base was removed, and the polysulfone chip containing the scaffolds was dehydrothermally cross-linked. A photograph of the mold, representative freezing profiles, and contrast-phase images of the resultant pore structures are shown in **Figure 4.2**.

Representative scaffolds from both sides of the array (aluminum, polysulfone) and both final freezing temperatures (-10, -40ºC) were selected for analysis. The scaffolds were embedded in glycolmethacrylate, serially sectioned in the transverse plane, and stained in order to visualize their struts. A linear intercept program was used to calculate a best-fit ellipse and determine a mean pore size and aspect ratio. The results are reported in **Table 4.1**. The aspect ratio of all four groups was close to 1, indicating a largely isotropic microstructure. The mean pore size for the aluminum section was 77 ± 16 μm (-10ºC) and 88 ± 28 μm (-40ºC). There was no statistically significant difference in mean pore size between these two variants. The mean pore size for the polysulfone section was 126 ± 32 μm (-10ºC) and 157 ± 47 μm (-40ºC). There was a statistically significant ($p < 0.0001$) difference in mean pore size between the aluminum-side variants and the polysulfone-side variants, for all possible final freezing temperature combinations.

Because the volumes of CG suspension used were quite small (on the order of 100 μL/node), it is expected that the rapid freezing time would outweigh any influences of final freezing temperature on scaffold mean pore size that are observed in macro-scale scaffolds (O'Brien, Harley et al. 2004). This would explain the unexpected result of the mean pore size being slightly greater for scaffolds frozen at a lower final freezing temperature. For scaffolds fabricated within

the array mold, the mean pore size appears to be entirely dependent on local heat transfer (through the base) during freezing, and not the final freezing temperature.

### 4.3 Cell Culture within Scaffold Array

In order to demonstrate the scaffold array's compatibility with existing high-throughput technologies, we devised an experiment to quantify cell number using a plate reader (Tecan, Switzerland). HL-1 cells stained with CellTracker Green CMFDA dye, seeded at three different cell densities (2E4, 5E4, 1E5 cells/scaffold) onto 1) scaffolds within the array, 2) scaffolds within a 96-well plate, or 3) directly onto a 96-well plate, and allowed to attach. The 96-well plate samples were read in the plate reader as normal; the scaffold array was placed on the lid of a 96-well plate and read using a 96-well plate protocol. The average fluorescent intensity for each sample was determined by taking multiple reads at different locations in each well, to account for heterogeneities in the scaffold groups. As expected, all three groups showed an overlapping linear relationship between intensity and cell seeding density (**Figure 4.3**).

### 4.4 Gradient Biomolecule Patterning

The ability to covalently tether biomolecules to CG scaffolds using benzophenone (BP) photolithographic chemistry has been previously reported in the literature (Martin, Caliari et al. 2011). However, this technique has not yet been used to create step-wise gradients within a biomaterial or series of biomaterials. BP can be excited by UV light to form a diradical that can react with a C-H bond from a nearby biomolecule to form a new C-C covalent bond. If BP does not react with a nearby molecule while it is in this excited state, it relaxes back to the ground state,

and can be re-excited with subsequent UV excitation. Since during processing BP is conjugated to free lysine side chains of the CG scaffold in dimethylformamide (DMF), an organic solvent that dissolves polysulfone, a second-generation array mold was created in order to utilize this BP patterning technique with the scaffold array. The second-generation array chip was made of 1/16" thick Macor, a machinable ceramic-glass composite, with the approximate node spacing/geometry of a 384-well plate (3.7 mm circular scaffold diameter). We used biotinylated concanavalin A as a model biomolecule for our proof-of-concept patterns.

The array chip was soaked in DMF containing 0.5 M N,N-Diisopropylethylamine (DIEA) and 20 mM Benzophenone-4-isothiocyanate (BP) for 2 days, then subjected to a series of washes to remove the any non-conjugated BP and residual DMF. The array was placed in water, then soaked in an aqueous solution of biotinylated concanavalin A (5 μg/mL) for 2 h prior to patterning. UV excitation of the BP was achieved using an argon ion laser (Coherent Innova 90-4, Laser Innovations). The laser power was adjusted to give a power of 20 $mW/cm^2$ at the scaffold surface. Each column of the array was exposed for a set amount of time (0, 3, 6, 9, 12 min) while the non-exposed regions were covered with a movable stage, in order to create a step-wise gradient of immobilized protein.

Following immobilization, the array was placed in 0.2% pluronic solution in PBS for 1.5 h under moderate shaking, rinsed multiple times in 1% BSA 0.1% Tween 20 blocking solution, then placed in 1% BSA overnight. The biotinylated concanavalin A was labeled with Alexa Fluor 488 conjugated streptavidin. The array was placed in PBS >2 h prior to imaging. Fluorescent

detection of immobilized protein was accomplished using a Typhoon Multimode Imager (GE). The mean fluorescent intensity of each scaffold was determined using the histogram feature of ImageJ (**Figure 4.4**). With increased exposure time, we see an increased degree of biomolecule immobilization. We are currently optimizing the patterning and washing processes to attain clearer gradients.

### 4.5 Figures and Tables

| Removable Base | Final Freezing Temperature | Average Pore Size | Aspect Ratio |
|---|---|---|---|
| **Aluminum** | -10$^{o}$C | 77 ± 16 μm | 0.944 ± 0.049 |
| **Polysulfone** | -10$^{o}$C | 126 ± 32 μm | 0.917 ± 0.065 |
| **Aluminum** | -40$^{o}$C | 88 ± 28 μm | 0.949 ± 0.056 |
| **Polysulfone** | -40$^{o}$C | 157 ± 47 μm | 0.887 ± 0.106 |

**Table 4.1**. Pore size/aspect ratio analysis obtained by stereology methods for scaffolds fabricated within the CG array mold. Pore size is indicated as average pore diameter ± standard deviation.

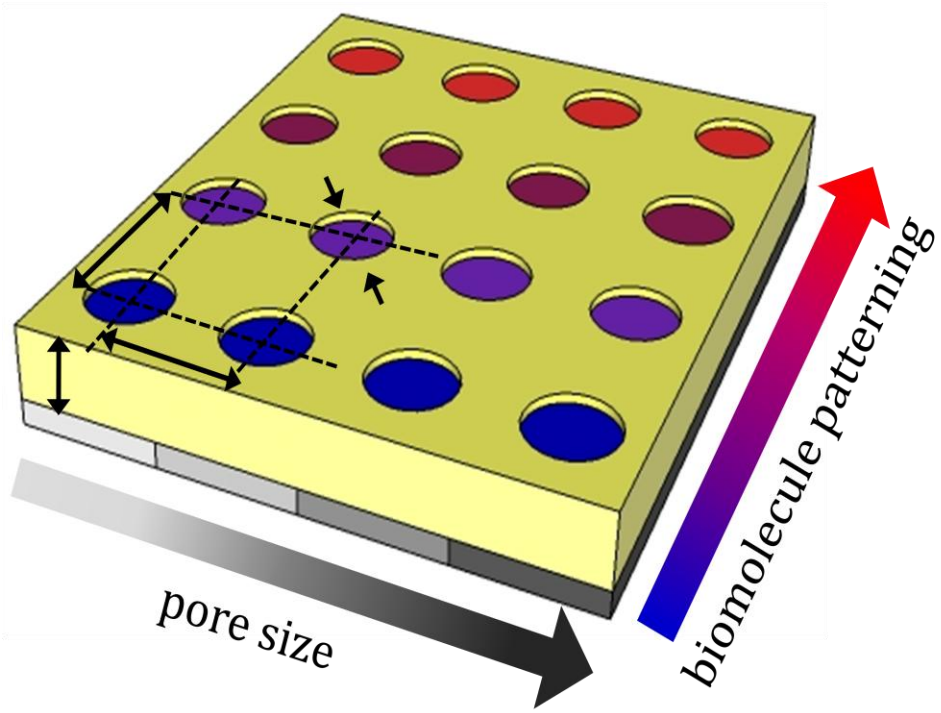



**Figure 4.1**. Schematic of array mold with varied pore size in one direction due to changes in mold base conductivity during freezing. In the other direction, biomolecule immobilization is varied in a gradient fashion, as to create a homologous series of microenvironments.

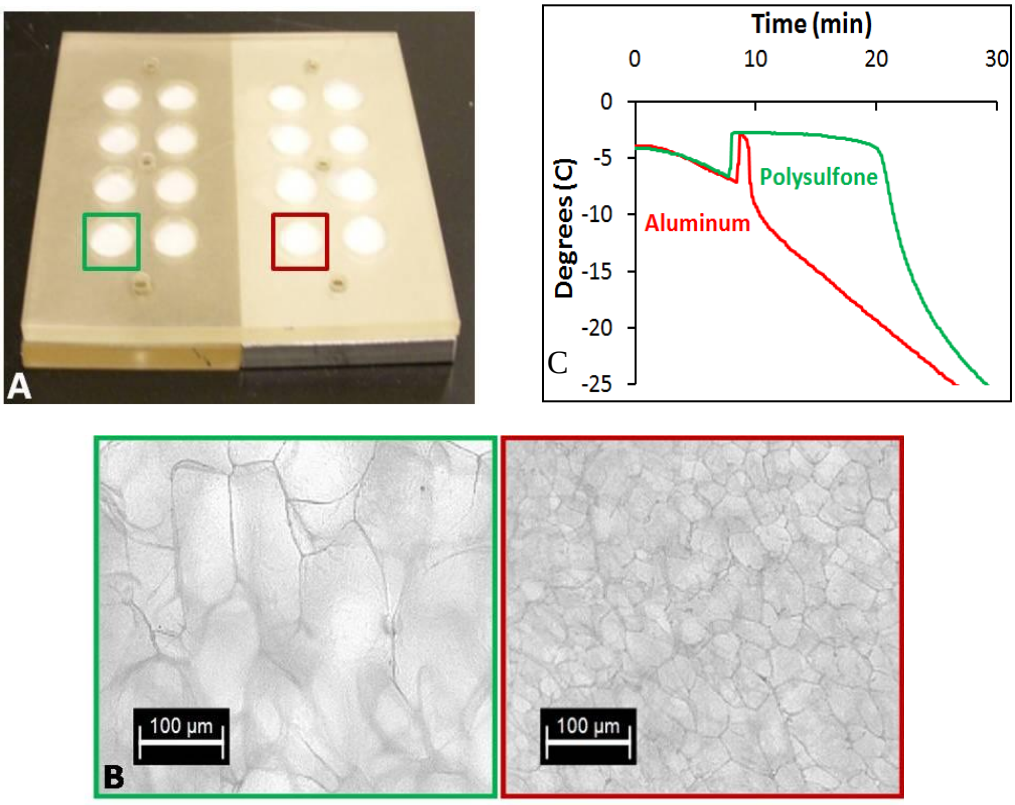



**Figure 4.2**. (A) Photograph of prototype scaffold array mold with disparate base thermal conductivities ($k_{aluminum}/k_{polysulfone} \approx 850$). (B) Freeze-dried slurries of constant chemical composition yield scaffolds with differing average pore sizes (phase contrast microscopy images: polysulfone *green*, aluminum *red*). (C) Representative solidification profiles for CG scaffolds freeze-dried in molds with aluminum (*red*) and polysulfone (*green*) bases. The ice crystal growth phase period is markedly shorter for the scaffolds fabricated in the mold with a base with a higher heat transfer coefficient (aluminum).

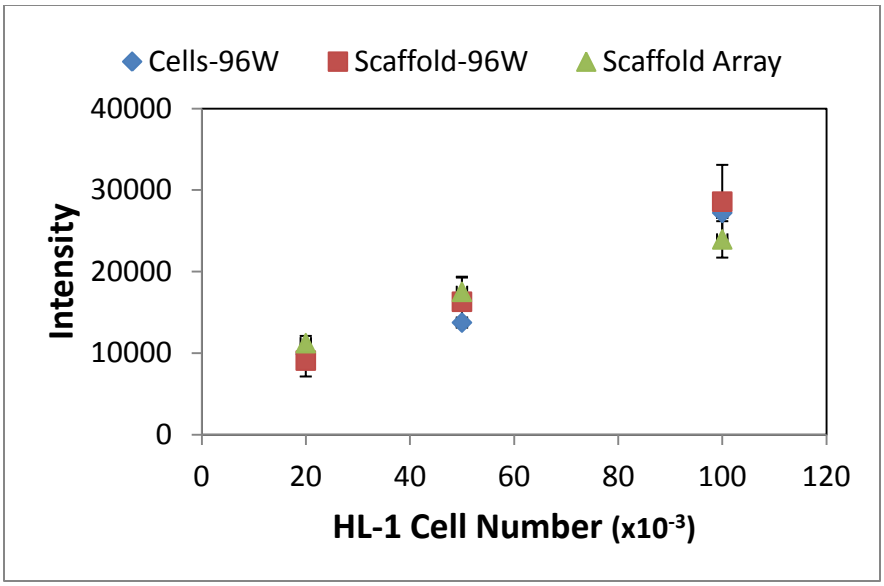
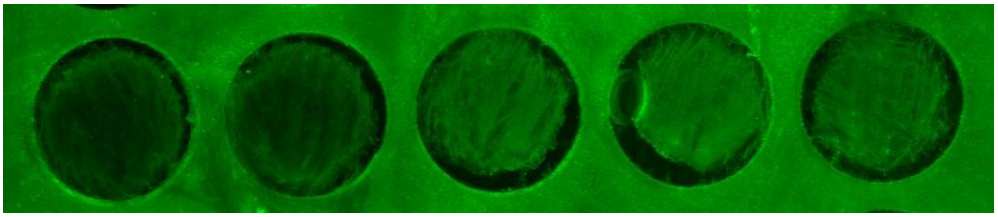



**Figure 4.3**. Fluorescent intensity of HL-1 cardiomyocytes stained with CellTracker Green CFMDA determined by plate reader analysis after 2 hours in culture: Cells cultured within a 96 well plate (blue diamonds); scaffolds cultured within 96 well plate (red squares); scaffolds cultured within CG array with 96-well plate geometry. To analyze the scaffold array, the entire array was placed on the lid of a 96-well plate; the scaffolds were *not* removed for analysis. At least n = 5 samples were analyzed per cell seeding density for the two controls; at least n = 3 samples were analyzed per cell seeding density for the scaffolds within the array. Graph x-axis indicates initial seeding density; error bars are ± standard error of the mean.



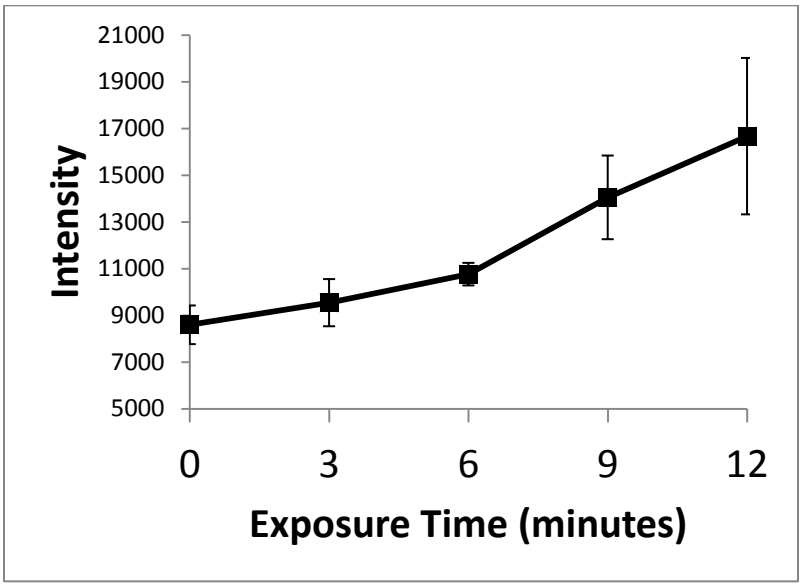



**Figure 4.4**. *Top*: Representative fluorescent image of a single row in the array patterned with a step-wise gradient of biotinylated Concanavalin A, with laser exposure times increasing from 0 minutes (left) to 3 minutes (right) in 45 second intervals. *Bottom*: Mean fluorescent intensity for scaffolds patterned with a step-wise gradient of biotinylated Concanavalin within n = 3 distinct rows of the array. Laser power was held constant, while exposure times were varied from 0 to 12 minutes in 3 minute intervals. Error bars indicate ± standard deviation.

# CHAPTER 5: CONCLUSIONS

Recognizing key factors of the native cardiac ECM (composition, collagen fiber anisotropy, sufficiently compliant mechanical properties), our efforts were directed to developing CG scaffolds for cardiac tissue engineering applications. The anisotropic scaffolds employed in this study had been previously used for tendon tissue engineering objectives, but had not yet been utilized for cardiac tissue engineering purposes. We were able to use type I CG scaffolds to identify specific microstructural properties that influenced HL-1 cell behaviors in 3D. These scaffolds are capable of supporting viable, metabolically active cardiac cell populations out to at least 14 days in culture.

Specifically, we saw that longitudinal pore anisotropy promoted increased beating for HL-1 cells cultured within these scaffolds. Not only did the anisotropic variants exhibit spontaneous beating as early as one day in culture (one day earlier than the isotropic control), but they demonstrated a statistically significantly higher proportion of center-localized beating for multiple days examined. Furthermore, this effect was dependent on pore size. Anisotropic variants with larger pore sizes had greater proportions of scaffolds with spontaneously beating regions than those with smaller pore sizes. Larger pore size is known to have other positive effects on scaffold bioactivity, including easier cell infiltration into the scaffold, which suggests that an aligned CG scaffold variant with larger pores (> 150 μm), may be most suited for cardiac tissue engineering applications.

We also showed that an anisotropic scaffold microstructure promoted increased HL-1 cardiomyocyte alignment in the longitudinal plane. Cell orientation appeared to be random in the isotropic control and the transverse planes of all variants examined. Promoting cell alignment is a desirable scaffold characteristic, as cells within the myocardium as well as the myocardium ECM itself are highly organized and anisotropic.

Future work could extend beyond the HL-1 model system and examine the spontaneous beating potential of primary cardiomyocytes within CG scaffold constructs. By using contrast-phase video footage of the scaffold struts, quantifying the beating frequency (Hz) would be possible. In addition, cardiomyocyte beating may be more easily detected via the use of calcium-sensitive fluorescent dyes. One key area to explore is the relative contributions of pore size and degree of anisotropy to the observed beating trends. Another interesting area to investigate would be disease state model culture conditions for these CG scaffold systems. As hypoxic conditions, associated with diabetes, are linked with changes in heart mechanical properties, it is likely that the observed beating trends would be altered. Determining gene expression profiles, including connexin 43 (gap junction protein associated with synchronized cardiomyocyte contraction) expression, for healthy and disease state models would enable an improved understanding of the regulatory mechanisms of cardiomyocytes in the diseased heart. The scaffold array technology described in Chapter 4 would enable the rapid analysis of multiple environmental cues on cardiac cell behavior, for both normal and diseased heart models.

# APPENDIX A: EXPERIMENTAL PROTOCOLS

## A.1 CG Suspension Preparation Protocol

*Reference:* (Yannas, Lee et al. 1989; O'Brien, Harley et al. 2004; Caliari and Harley 2011; Gonnerman, McGregor et al. in preparation)

*Reagents*

- Collagen from bovine Achilles tendon (Sigma-Aldrich C9879); store at 4ºC
- Chondroitin sulfate sodium salt from shark cartilage (Sigma-Aldrich C4384); store at 4$^{o}$C
- Glacial acetic acid (Sigma-Aldrich 71251)
- Ethylene glycol (VWR BDH1125-4LP)
- Deionized water

*Supplies and equipment*

- Recirculating chiller (Fisher Isotemp Model 900)
- Rotor-stator (IKA 0593400)
- Disperser (IKA 3565001)
- Jacketed beaker (Ace Glass 5340-115)
- Freeze-dryer (VirTis Genesis)
- Beakers
- Parafilm

*Procedure*

*This procedure describes how to make 300 mL of 0.5% CG suspension. Scale collagen and GAG content appropriately to create different volumes of suspension.

1) Fill recirculating chiller with a 50/50 mix of ethylene glycol and deionized water, making sure that the cooling coils are completely immersed in the liquid. Set the recirculating chiller to 4$^{o}$C.
2) Attach recirculating chiller to jacketed beaker so that the coolant enters at the jacketed beaker's base and exits at the beaker's top. Allow for the temperature to equilibrate to 4$^{o}$C, about 30 minutes. Maintaining this temperature is important, as it will prevent the collagen from denaturing during the blending process.
3) Prepare a 0.05 M solution of acetic acid by adding 0.87 mL of glacial acetic acid to 300 mL of deionized water.
4) Weigh 1.5 g of collagen and add to the jacketed beaker.
5) Pour 250 mL of the 0.05 M acetic acid into the jacketed beaker.
6) Assemble the rotor-stator and attach it to the disperser. Lower the rotor-stator into the suspension. The rotor-stator should be vertical and centered in the beaker.

7) Blend the suspension at 15,000 rpm for 90 min at 4$^{o}$C. The height of the rotor-stator may need to be adjusted during the blending process: If the rotor-stator is positioned too high, the holes on its side will be visible; if it is too low, the suspension will bubble excessively. Periodically check to see if the rotor-stator is clogged with collagen; remove clogs with a spatula as needed.
8) Add 50 mL of 0.05 M acetic acid to a 50 mL centrifuge tube. Weigh out 0.133 g of chondroitin sulfate (GAG) and add to the centrifuge tube. Vortex until the GAG is fully dissolved. Let the GAG solution rest in the refrigerator (4$^{o}$C) for at least 10 minutes.
9) Add the GAG solution drop-wise to the collagen suspension while it is being mixed at 15,000 rpm at 4$^{o}$C. Periodically manually stir in any GAG that remains on the surface of the suspension using a spatula. It may be necessary to stop and unclog the rotor-stator with a spatula during this process.
10) Once all of the GAG solution has been added, blend at 15,000 rpm for 90 min at 4$^{o}$C. Periodically check to ensure the rotor-stator is lowered to the correct depth, as the suspension will gradually become less viscous and creep up the sides of the jacketed beaker. Periodically check to see if the rotor-stator is clogged; remove clogs with a spatula as needed.
11) Store the suspension for at least 18-22 h at 4°C.
12) Degas the suspension to remove any air bubbles prior to use. It is recommended to degas approximately 20 mL at a time, until the solution starts to boil. To minimize suspension loss during the degassing process, cover the beaker with slit Parafilm.
13) Store the suspension at 4$^{o}$C. Periodically check the CG suspension; if not homogenous, re-blend at 15,000 rpm for at least 30 min at 4$^{o}$C.

## A.2 Aligned CG Scaffold Fabrication Protocol

*Reference:* (Caliari and Harley 2011; Gonnerman, McGregor, et al. in preparation)

*Reagents*

- CG suspension; store at 4°C
- Welch DirecTorr Gold synthetic pump oil (Fisher 01-184-105)

*Supplies and equipment*

- Freeze-dryer (VirTis Genesis)
- Teflon-copper (CuTef) freeze-drying mold
- Beakers
- Parafilm
- Aluminum foil

*Procedure*

*This procedure describes the fabrication of 15 mm tall aligned scaffolds. Check that oil is clean (clear, not yellowed) before and after each freeze-dryer run, replacing when necessary. It is easiest to replace the oil just after a run, when the oil is still warm.

1) Load and run the "degas" program, which lowers the freeze-dryer condenser temperature to -70°C and the shelf temperature to 4°C. When the condenser is <-50°C, one can cancel the cycle and begin degassing. This is done to avoid contaminating the vacuum pump.
2) Degas the CG suspension in a beaker (covered in Parafilm with small slits) by pulling vacuum inside freeze-dryer. Degas just to the boiling point to remove all air bubbles.
3) Begin to cool the freeze-dryer shelves by running 'Tf = xx C shelf cool' program where xx is the desired freezing temperature (-10, -40, or -60°C).
4) When the desired shelf temperature has been reached, pipette 920 μL of suspension into each hole (8 mm diameter) of the Teflon-copper freeze-drying mold.
5) Cancel the shelf cool program and quickly place the freeze-dryer mold on the pre-cooled shelf. Quickly close the freeze-dryer door and run the program 'Aligned Tf = xx' where xx is the desired freezing temperature (-10, -40, or -60°C).
   A typical schedule is shown below for the fabrication aligned scaffold with a freezing temperature of -10°C.

| Step | Temperature, °C | Time, minutes | Ramp/ Hold | Vacuum level, torr | PCM |
|---|---|---|---|---|---|
| Freezing hold | -10 | 60 | H | ~600 | N/A |
| Drying ramp | 0 | 10 | R | 0.2 | 150 |
| Drying hold | 0 | 5 | H | 0.2 | 1 |
| Additional drying | 0 | 60 | H | 0.2 | 0 |
| Storage ramp | 20 | 20 | R | 0.2 | 0 |
| Storage hold | 20 | indefinite | H | 0.2 | 0 |

6) Once the program has reached the storage hold stage, the program can be cancelled and scaffolds can be removed from the freeze-dryer.

7) Allow scaffolds to sit in mold at room temperature for at least 1 hour before carefully removing them with forceps and placing in an aluminum foil pouch. Label pouch with name, collagen type, collagen concentration, freeze date, freeze temperature, and any other relevant notes.

## A.3 Isotropic CG Scaffold Fabrication Protocol

*Reference:* (Martin, Caliari, et al. 2011; Gonnerman, McGregor, et al. in preparation)

*Reagents*

- CG suspension; store at 4°C
- Welch DirecTorr Gold synthetic pump oil (Fisher 01-184-105)

*Supplies and equipment*

- Freeze-dryer (VirTis Genesis)
- Aluminum, polysulfone tray molds (3"x3")
- Beakers
- Parafilm
- Aluminum foil
- Tweezers

*Procedure*

*This procedure describes the fabrication of 3 mm tall scaffold sheets. Check that oil is clean (clear, not yellowed) before and after each freeze-dryer run, replacing when necessary. It is easiest to replace the oil just after a run, when the oil is still warm.

1) Load and run the "degas" program, which lowers the freeze-dryer condenser temperature to -70°C and the shelf temperature to 4°C. When the condenser is <-50°C, one can cancel the cycle and begin degassing. This is done to avoid contaminating the vacuum pump.
2) Degas the CG suspension in a beaker (covered in Parafilm with small slits) by pulling vacuum inside freeze-dryer. Degas just to the boiling point to remove all air bubbles.
3) Add 24.25 mL of CG suspension to a 3x3 tray mold, ensuring that the suspension reaches the corners. Push any bubbles or unblended collagen to the edge using tweezers.Open freeze- dryer door, place mold on center of shelf. Quickly close the freeze-dryer door and run the program 'Tf-xx No Hold' where xx is the desired freezing temperature (-10, -40, or -60°C). A typical schedule is shown below for the constant cooling fabrication method with a final freezing temperature of -40°C.

| **Step** | **Temperature, °C** | **Time, minutes** | **Ramp/ Hold** | **Vacuum level, torr** | **PCM** |
|---|---|---|---|---|---|
| Initial hold | 20 | 5 | H | ~600 | N/A |
| Freezing ramp | -40 | 60 | R | ~600 | N/A |
| Freezing hold | -40 | 120 | H | ~600 | N/A |
| Drying ramp | 0 | 40 | R | 0.2 | 150 |
| Drying hold | 0 | 5 | H | 0.2 | 1 |
| Additional drying | 0 | 60 | H | 0.2 | 0 |
| Storage ramp | 20 | 20 | R | 0.2 | 0 |
| Storage hold | 20 | indefinite | H | 0.2 | 0 |

4) Once the program has reached the storage hold stage, the program can be cancelled and the array can be removed from the freeze-dryer.

5) Gently remove scaffold by lifting from corner with tweezers. Place scaffold in puffed aluminum pouch. Label pouch with name, collagen type, collagen concentration, freeze date, freeze temperature, and any other relevant notes. Clean mold by rubbing with soapy water; use 0.05 M acetic acid to remove collagen residue. Do not use cleaning brushes.

## A.4 CG Array Scaffold Fabrication Protocol

*Reference:* (Gonnerman, Turgeon, et al. in preparation)

*Reagents*

- CG suspension; store at 4°C
- Welch DirecTorr Gold synthetic pump oil (Fisher 01-184-105)

*Supplies and equipment*

- Freeze-dryer (VirTis Genesis)
- Macor array chip with removable aluminum and polysulfone bases
- Beakers
- Parafilm
- Putty knife
- Aluminum foil
- Glass petri dish

*Procedure*

*This procedure describes the fabrication of scaffold arrays. Check that oil is clean (clear, not yellowed) before and after each freeze-dryer run, replacing when necessary. It is easiest to replace the oil just after a run, when the oil is still warm.

1) Load and run the "degas" program, which lowers the freeze-dryer condenser temperature to -70°C and the shelf temperature to 4°C. When the condenser is <-50°C, one can cancel the cycle and begin degassing. This is done to avoid contaminating the vacuum pump.
2) Degas the CG suspension in a beaker (covered in Parafilm with small slits) by pulling vacuum inside freeze-dryer. Degas just to the boiling point to remove all air bubbles.
3) Add ~30 μL of CG suspension to each node of the array. Once all nodes have been filled, add ~2 mL of CG suspension to the top of the array, forming a bubble-like layer that bridges both halves of the chip. Using the putty knife, gently scrape away the excess CG suspension, leaving a little residual to form a thin film on the surface.
4) Open freeze-dryer door, place mold on center of shelf. Quickly close the freeze-dryer door and run the program 'Tf-xx No Hold' where xx is the desired freezing temperature (-10, -40, or -60°C). A typical schedule is shown below for the constant cooling fabrication method with a final freezing temperature of -40°C.

| Step | Temperature, °C | Time, minutes | Ramp/ Hold | Vacuum level, torr | PCM |
|---|---|---|---|---|---|
| Initial hold | 20 | 5 | H | ~600 | N/A |
| Freezing ramp | -40 | 60 | R | ~600 | N/A |
| Freezing hold | -40 | 120 | H | ~600 | N/A |
| Drying ramp | 0 | 40 | R | 0.2 | 150 |
| Drying hold | 0 | 5 | H | 0.2 | 1 |
| Additional drying | 0 | 60 | H | 0.2 | 0 |
| Storage ramp | 20 | 20 | R | 0.2 | 0 |
| Storage hold | 20 | indefinite | H | 0.2 | 0 |

5) Once the program has reached the storage hold stage, the program can be cancelled and the array can be removed from the freeze-dryer.
6) Carefully disassemble mold by unscrewing one base at a time, taking care not to unnecessarily tear the collagen film layer. Remove bases one at a time with a horizontal sliding motion. Place Macor chip in a glass petri dish, and place petri dish in an aluminum pouch. Label pouch with name, collagen type, collagen concentration, freeze date, freeze temperature, and any other relevant notes.
7) Clean mold by rubbing with soapy water; use 0.05 M acetic acid to remove collagen residue. Do not use cleaning brushes.

## A.5 DHT Crosslinking Protocol

*Reference:* (Yannas, Lee et al. 1989; Harley, Leung et al. 2007)

*Supplies and equipment*

- Sterile air filter (Millipore SLGP033RS)
- Vacuum oven (Wlech Vacuum, Fisher 13-262-52)
- Welch DirecTorr Gold synthetic pump oil (Fisher 01-184-105)

*Procedure*

*Note: Periodically check vacuum pump oil levels. Change oil at least once every 6-12 months. Change sterile air filter on 'Purge' line regularly.

1) Turn on vacuum oven and set the temperature to 105$^{o}$C.
2) Once vacuum oven has reached temperature set point, place scaffolds in opened aluminum pouches carefully inside the oven. Close the oven door.
3) Close the 'Purge' valve, located on the lower right face of the vacuum oven. Completely open the 'Vacuum' valve.
4) Turn on the vacuum pump and make sure vacuum is pulled to a sufficiently low level (< 1 in Hg). Allow scaffolds to crosslink for 24 hours.
5) After crosslinking is complete, turn off the vacuum pump, close the 'Vacuum' valve, open the 'Purge' valve, then carefully remove scaffolds from the oven. Quickly seal the aluminum pouches, taking care to ensure that the aluminum pouches are sufficiently "puffed" so that the scaffolds will not be crushed during storage. Store sealed pouches with scaffolds (now sterile) in desiccator until time of use.

## A.6 Scaffold Cutting and EDAC Crosslinking Protocol

*Reference:* (Olde Damink, Dijkstra et al. 1996; Harley, Leung et al. 2007; Caliari and Harley 2011; Gonnerman, McGregor, et al., in preparation)

*Reagents*

- 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDAC, Sigma-Aldrich E7750); store at -20ºC
- N-hydroxysulfosuccinimide (NHS, Sigma-Aldrich H7377); store in desiccators
- Sterile phosphate-buffered saline (PBS)
- 200 proof (100%) ethanol

*Supplies and equipment*

- 6-well plates (Fisher 08-772-1B
- 50 mL centrifuge tubes (Fisher 14-432-22)
- Syringe and syringe filter (Fisher 148232A)
- MTS 2/4 digital microtiter shaker (IKA 3208001)
- Dual range balance (Mettler Toledo XS105)
- Razor blades
- 6 mm biopsy punches (Fisher NC9551417)
- Pasteur pipettes

*Procedure*

* Note: all steps should be performed in the laminar flow hood unless otherwise noted.

1) Prior to cutting scaffolds, ensure that all materials (including gloves) are completely dry.
2) Cut aligned scaffold samples to be crosslinked using a razor blade. With a gentle sawing motion, cut each aligned scaffold into quarters, using the top of a sterile well plate as a cutting board. Discard the top and base quarters. Place cut scaffolds in labeled pre-weighed 50 mL conical tubes.
3) Cut isotropic scaffolds from scaffold sheets using a biopsy punch. Ensure that the biopsy punch is oriented perpendicular to the scaffold sheet. Holding the top of the biopsy punch, gently spin the biopsy punch downward to cut through the sheet, applying pressure at the end only if necessary. If the scaffold remains lodged in the biopsy punch, gently poke it out using a Pasteur pipette. Place cut scaffolds in labeled pre-weighed 50 mL conical tubes.
4) Weigh scaffolds prior to hydration.
5) Hydrate cut pieces in 100% ethanol overnight.
6) Rinse pieces several (>3) times in PBS, then let soak in PBS for 24 hours prior to crosslinking.
7) Determine the EDAC and NHS concentrations to be used in the crosslinking solution. The calculations shown below are done with a 5:2:1 EDAC:NHS:COOH molar ratio, where COOH is carboxylic acid groups in CG material based on a conversion factor of 1.2 mmol COOH per gram of collagen (Olde Damink, Dijkstra et al. 1996). The mass of EDAC and NHS required can be calculated as follows:

$$M_{EDAC} = M_{scaffold}\left(\frac{0.0012\ mol_{COOH}}{g_{collagen}}\right)\left(\frac{5\ mol_{EDAC}}{1\ mol_{COOH}}\right)\left(\frac{191.7\ g_{EDAC}}{1\ mol_{EDAC}}\right)$$

$$M_{NHS} = M_{scaffold}\left(\frac{0.0012\ mol_{COOH}}{g_{collagen}}\right)\left(\frac{2\ mol_{NHS}}{1\ mol_{COOH}}\right)\left(\frac{116.0\ g_{NHS}}{1\ mol_{NHS}}\right)$$

8) Hydrate scaffolds in 100% ethanol overnight.
9) Mix the EDAC and NHS in PBS. Approximately 1 mL of solution will be needed per scaffold piece (6-8 mm diameter, 3-5 mm thick).
10) In the laminar flow hood, sterile filter the solution and add to 6-well plates. One can crosslink up to 6 scaffolds per well. If volume is insufficient to cover scaffolds, add additional PBS, keeping the volume constant for all wells.
11) Add scaffolds to crosslinking solution and place well plate on digital microtiter shaker in 37$^{o}$C incubator. Allow scaffolds to crosslink under moderate shaking for 30 minutes. Crosslinking time should be increased for less permeable constructs and higher solids content scaffolds.
12) Remove EDAC/NHS solution and rinse scaffolds in sterile PBS under moderate shaking for 10-15 minutes.
13) Remove PBS wash solution and replace with fresh PBS. Rinse under moderate shaking for an additional 30-45 minutes.
14) Store in fresh sterile PBS at 4$^{o}$C until use.

## A.7 Step-Wise Gradient Patterning of ConA-Biotin onto CG Scaffold Array Using Benzophenone Photochemistry Protocol

*Reference:* (Martin, Caliari et al. 2011; Gonnerman, Turgeon, et al. in preparation)

*Reagents*

- Dimethylformamide (DMF)
- Benzophenone-4-isothiocyanate (BP); store at -20ºC, desiccated (Sigma B6931)
- N,N-Diisopropylethylamine (DIEA); store at room temperature (Sigma 03440)
- Biotinylated Concanavalin A (Con A) stock, 5 mg/mL stock; store at -80ºC (Sigma B-1005)
- 100% (200 proof) ethanol
- Streptavidin Alexa FluorA 488 conjugate; store at 4ºC (Invitrogen S32354)
- PBS, without $Ca^{2+}$ and $Mg^{2+}$
- 1% bovine serum albumin (BSA) and 0.1% Tween 20 in PBS
- 2% bovine serum albumin (BSA) in PBS

*Supplies and equipment*

- Argon ion laser (Coherent Innova 90-4, Laser Innovations, Santa Paual, CA)
- Scaffold microarray with Macor upper chip
- Typhoon Multimode Imager (GE)
- Belly Dancer Shaker (Stovall Life Science)
- ImageJ Software
- Aluminum foil

*Procedure*

*Note: This method is light-sensitive. Ensure that petri dishes are covered in aluminum foil (except during laser exposure).
*Note: DMF will dissolve plastics. Use glass petri dishes up to blocking step.

1) In a glass petri dish, hydrate scaffolds in DMF with BP and DIEA (50 mg BP/10 mL DMF; 500 µL DIEA/10 mL DMF). Let stand for 48 h.
2) Rinse scaffolds in DMF 2-3 times. Let sit in fresh DMF overnight. Place in fresh DMF the following morning. If necessary, keep rinsing in DMF until scaffolds appear white (not yellow).
3) Rinse scaffolds with 100% ethanol for at least 1 h (can go up to 2 days).
4) Rinse scaffolds with DI water 2-3 times. Soak in DI water >1 h.
5) Soak scaffolds in biotin-Con A at a ratio of 10 µL biotin-Con A stock/10 mL DI water. At least 10 mL water will be necessary to cover array, 20 mL is preferred. Soak in protein solution for 2 h.
6) While covering non-exposed regions with moveable stage, expose scaffolds to UV-laser beam in row-dimensioned rectangles, varying exposure time from 0 to 12 minutes in 3 minute intervals (or other desired intervals/maximum exposre times), with the power held constant at ~20 $mW/cm^2$. The notched corner of the array indicates a row exposure time of 0 minutes.

7) Rinse array in 0.2% pluronic for 1-1.5 hours (to deactivate BP), under moderate shaking.
8) Rinse array in PBS.
9) Place array in 1% BSA and 0.1% Tween 20 in PBS for 1 h under moderate shaking. Rinse again in fresh 1% BSA and 0.1% Tween 20 in PBS for 1 h under moderate shaking.
10) Block in 1% BSA and 0.1% Tween BSA in PBS overnight on shaker.
11) Rinse multiple times with PBS prior to staining.
12) Make up ~15 mL of staining solution (1 μL AlexaFluor 488 to 2 mL of 2% BSA in PBS; vortex lightly). Place array in staining solution. Let sit, at 4ºC overnight.
13) Wash in PBS for 1 h at 4ºC. Replace with fresh PBS and let sit an additional 2 h.
14) Image gradient using Typhoon Multimode Imager. Perform a test scan with poor resolution prior to imaging to test laser power. Image will be grayscale with darker regions indicating greater fluorescence. Red regions indicate oversaturation.

Settings: Fluorescent intensity scan
Filters: Excitation green (532 nm); Emission 526 nm
PMT: 600 (adjust if needed)
Resolution: 25 μm

## A.8 Scaffold Embedding Protocol

*Reference:* (O'Brien, Harley et al. 2004; O'Brien, Harley et al. 2005, Caliari and Harley 2011)

*Reagents*

- 100 mL JB-4A embedding solution with catalyst (100 mL); store at 4ºC for up to 1 week
    - 100 mL JB4A embedding solution (monomer) (Polysciences 0226A-800)
    - 1.25 g JB-4 catalyst (benzoyl peroxide, plasticized); store at 4ºC
- JB-4B embedding solution (accelerator) (Polysciences 02618-12)
- 100% ethanol

*Supplies and equipment*

- Polyethylene molding cup trays (Polysciences 16643A-1)
- JB-4 plastic block holders (Polysciences 15899-50)
- DryFast vacuum pump (Welch Vacuum 2014B-01)
- Pyrex desiccator (Fisher 08-626B)
- Plastic Pasteur pipettes
- 6-well plates (Fisher 08-772-1B)
- Chemical fume hood
- Razor blades
- 6 mm biopsy punches (Fisher NC9551417)
- Round-tip forceps (VWR 82027-394)

*Procedure*

*Note: All steps should be performed in a chemical fume hood.

1) Cut scaffold pieces to be analyzed using a razor blade (aligned variants) or a biopsy punch (isotropic variants). Biopsy punches should not exceed 6 mm in diameter, or the scaffold will deform slightly during the embedding process. Use a razor blade to obtain longitudinal cross-sections, if desired.
2) Place samples in 6-well plates and hydrate in 100% ethanol under vacuum inside desiccator overnight, or until no bubbles remain.
3) Add hydrated samples to JB-4A embedding solution with catalyst. Hold under vacuum inside desiccator at 4ºC for 24 h.
4) Transfer samples to fresh JB-4A solution with catalyst. Hold under vacuum inside desciccator at 4ºC for 48 h.
5) Label plastic stubs using pencil (lab marker is erased by JB-4 solution).
6) Mix 25 mL of JB-4A embedding solution with catalyst with 1 mL of JB-4B solution and pipette ~3.5 mL into each well of the embedding mold.
7) Place each sample into a well. The JB-4 solution will polymerize quickly (< 30 min), so use tweezers to ensure samples stay in the proper orientation.
8) Monitor the polymerization of the JB-4 solution by gently poking with tweezers. Brown streaks will form in the tweezers' wake as the mixture begins to harden. Samples will often polymerize at slightly different rates, so monitor all samples carefully.

9) Place one labeled plastic stub into each well once the JB-4 mixture has become sufficiently viscous that the stubs don't completely sink.
10) Transfer embedding mold to 4ºC refrigerator. Hold at 4ºC overnight to allow the polymerization reaction to complete.
11) To remove the embedded samples from the mold, use the 'pointed' end of a 15 mL conical tube, and push down on the mold at multiple points around the edge of each sample to pop it out.
12) If residual "sticky" polymer remains on the edges, let dry plastic stub-side down in the chemical fume hood overnight.
13) Store samples at 4ºC until use.

## A.9 Scaffold Pore Size Analysis: Aniline Blue Staining, Image Acquisition, and Linear Intercept Analysis Protocol

*Reference:* (O'Brien, Harley et al. 2004; O'Brien, Harley et al. 2005, Caliari and Harley 2011; Weisgerber, et al. *in preparation*)

*Reagents*

- Aniline blue solution (100 mL)
  - 2.5 g aniline blue (Fisher AC40118-0250)
  - 2 mL glacial acetic acid (Sigma-Aldrich 71251)
  - 100 mL deionized water
- 1% acetic acid (100 mL)
  - 1 mL glacial acetic acid (Sigma-Aldrich 71251)
  - 99 mL DI water
- 95% ethanol (190 proof)
- 100% ethanol (200 proof)
- Permount mounting medium (Fisher SP15-100)

*Supplies and equipment*

- Contrast-phase optical microscope with camera (Leica Microsystems DMIL LED with DFC295 camera)
- Scion Image analysis software
- 150 mL beakers
- Cover slips
- Chemical fume hood

*Procedure*

*Aniline blue staining procedure*

1) Obtain slides of serially sectioned embedded scaffolds from histologist.
2) Dip slides in aniline blue solution for 2-4 minutes. Stain ~12 slides at a time.
3) Places slides in 1% acetic acid for 1 min.
4) Dip each slide several times in 95% ethanol until most of the background staining goes away.
5) Dip each slide several times in 95% ethanol to complete rinse and place on paper towel to dry for ~1 h.
6) Verify that samples have not been over-washed. If necessary, re-stain with aniline blue.
7) In chemical fume hood, add 1 drop of Permount directly on top of each cross-section to be analyzed (typically 2 per slide). Firmly press cover slip onto the slide, pushing from the center outwards to ensure that no air bubbles are introduced. It is easy to contaminate samples with excess glue, so check gloves for glue frequently.
8) Allow slides to dry in chemical fume hood for 24 h before further analysis.

*Image acquisition procedure*

1) Visualize embedded, sectioned, and stained scaffold samples using contrast-phase optical microscope (10x objective, open filter).
2) Acquire three tiff images for each cross-section analyzed. Avoid capturing images containing 'ribbing' artifacts from sectioning, bubbles, or unwashed aniline blue stain.

*Linear intercept procedure*

*Note: This process, including choosing appropriate threshold values, can be automated as described in Weisgerber et al (in preparation).

1) Transfer images to be analyzed to a folder with a short path length from the C drive.
2) Using Scion Image:
   Open > [filename.tif]
   Edit > Invert
   Options > Threshold
      Adjust the threshold value to optimize scaffold strut visualization. Small speckling artifacts (< 5 pixels) will not be detected by the pore size analysis macro.
   Process > Binary > Make Binary
      Save the edited tiff file.
   Analysis > Set Scale > 867 pixels per mm
      (Valid only for 10x magnification).
   Special > Load Macros
      Load the 'pore characterization macros Steven' file found on the desktop.
3) Select a region of the image to be analyzed with the oval drawing tool (the larger the image the region the better).
4) Special > Linear Intercept
   Special > Plot Intercepts
      The macro calculates the best fit ellipse with parameters C0, C1, and C2.
5) Transfer the values for C0, C1, and C2 to a spreadsheet.
6) Calculate the minor (a) and major (b) axes of the best fit ellipse using the following equations:

$$a = \frac{1}{\sqrt{C_0}+\sqrt{C_1^2+C_2^2}} \qquad b = \sqrt{\frac{\sqrt{C_1^2+C_2^2}}{\sqrt{C_0}\sqrt{C_1^2+C_2^2}+C_2^2-C_1^2}}$$

$$aspect\ ratio\ = \frac{b}{a}$$

7) The average pore size (d) is calculated using the following equation. The correction factor of 1.5 is used to account for the fact that the pores were not sectioned through their maximal cross-section, and the 2 converts from radius to diameter.

$$d\ = 2 * 1.5\sqrt{\frac{a^2+b^2}{2}}$$

## A.10 Incubator Disinfection Protocol

*Reagents*

- Steris staphene spray (Fisher 14-415-15)
- 70% ethanol

*Procedure*

1) Close valves to $CO_2$ tanks and turn off the incubator.
2) Prepare the sterile hood by covering the inside with bench-coat.
3) Cover the chemical fume hood with fresh bench-coat.
4) Disassemble all moveable parts (e.g. shelves) from the incubator chamber. Place parts in chemical fume hood. Spray all parts with staphene. Let stand 15 minutes.
5) Meanwhile, spray the inside of the incubator with staphene. Let stand 15 minutes with the incubator door cracked open ~2 in.
6) Spray all internal parts of the incubator in the chemical fume hood with 70% ethanol and wipe off the excess staphene with paper towels. Spray each part generously with 70% ethanol again and place in sterile hood to dry. Do not wipe anything down. Allow to air dry for 15-30 minutes.
7) Spray down the inside of the incubator with 70% ethanol. Wipe off the excess staphene with paper towels.
8) Spray down the inside of the incubator with 70% ethanol and allow to dry for 15-30 minutes; do not wipe anything down.
9) Reassemble all internal pieces of the incubator, taking care to move each piece from the sterile hood to the incubator as quickly as possible.
10) Spray the inside of the incubator with 70% ethanol.
11) Shut the door and allow all parts to dry; do not wipe anything down.
12) Turn on the incubator power and open the valves on the $CO_2$ tanks. Allow the incubator to ventilate with the $CO_2$ on for 24 h prior to use.

## A.11 HL-1 Cell Culture Protocol

*Reference*: (White, Constantin, et al. 2004; Claycomb, Lanson, et al. 1998; Gonnerman, McGregor, et al. in preparation)

*Reagents*

- Complete Claycomb Medium; cover in aluminum foil (is light sensitive) and store at 4$^{o}$C
    - 87 mL Claycomb Medium (Sigma-Aldrich 51800C ); cover in aluminum foil and store at 4$^{o}$C
    - 10 mL fetal bovine serum (Sigma-Aldrich 12103C, Batch 8A0177, OR Sigma-Aldrich F2442, Batch 058K8426); store at -20$^{o}$C
    - 1 mL pen-strep (Invitrogen 15140-122); store at -20$^{o}$C
    - 1 mL L-glutamine (Invitrogen 25030-081); store at -20$^{o}$C
    - 1 mL Norepinephrine stock solution; store at -20$^{o}$C
      **Safety note: Norepinephrine is highly toxic. Wear appropriate PPE and use a chemical fume hood when weighing out powder.**
        - Make up 100 mL of 30 mM ascorbic acid by adding 0.59 g ascorbic acid (Wako 014-04801) to 100 mL of sterile deionized water.
        - In chemical fume hood, add 80 mg norepinephrine (Sigma-Aldrich A0937) to 25 mL of 30 mM ascorbic acid.
        - Filter sterilize using a 0.22 μm syringe filter (Fisher SLMP025SS)
        - Aliquot in 1 mL volumes and store in sterile microcentrifuge tubes at -20$^{o}$C.
        - Norepinephrine should be made up fresh monthly.

      Note: If there are problems obtaining the medium or FBS, refer to Reservation # 21025944.
- Trypsin-EDTA 0.05% (Invitrogen 25300-062); store at -20$^{o}$C
- Sterile phosphate-buffered saline without $Ca^{2+}$ and $Mg^{2+}$ (PBS)
- Gelatin from bovine skin (Sigma-Aldrich G9391); desiccate
- Fibronectin (Sigma-Aldrich F-1141); store at 4$^{o}$C
- Fetal bovine serum (FBS) (Sigma-Aldrich 12103C, Batch 8A0177, OR Sigma-Aldrich F2442, Batch 058K8426); store at -20$^{o}$C
- Dimethyl Sulfoxide (DMSO)

*Supplies and equipment*

- 500 mL media bottles
- 100 mL media bottles
- Sterile serological pipettes (5 mL, 10 mL, 25 mL) (Fisher 13-678-11D, 13-678-11E, 13-678-11)
- Conical centrifuge tubes (15 mL: Fisher 05-527-90, 50 mL: Fisher 14-432-22)
- Contrast-phase optical microscope (Leica Microsystems)
- Round-bottom cryovials, 2 mL (Corning)

*Procedure*

*Pre-coating flasks with gelatin/fibronectin*

1) Weigh out 0.1 g of gelatin and place into a 500 mL media bottle.
2) Add 500 mL of DI water to the bottle and autoclave. The gelatin will go into solution during autoclaving (0.02% gelatin solution). Let cool to room temperature.
3) Dilute 1 mL of fibronectin into 199 mL of 0.02% gelatin. Swirl gently to mix, and immediately aliquot 6 mL per 15 mL conical tube. Freeze aliquots at -20$^{o}$C.
4) To coat culture flasks, thaw aliquoted gelatin/fibronectin and add 2 mL/T25 flask (6 mL/T75 flask). Ensure that coating covers entire base. Let incubate at 37$^{o}$C overnight.
5) Remove gelatin/fibronectin via aspiration just prior to adding cells to the flask.

*Culturing cells*

1) Cells should be cultured on gelatin/fibronectin coated flasks at 37$^{o}$C and 5% $CO_2$.
2) Cells should be fed complete culture medium daily (5 mL/T25 flask, 15 mL/T75 flask), and split 1:3 when confluent.
3) To avoid feeding cells on weekends, feed cells 10 mL/T25 flask on Friday afternoon; feed again Monday morning.

*Passaging cells*

*Note: It is recommended that cells be passaged only after reaching full confluence (for a 1:3 split, this typically takes 4 days). A few cells should be beating in confluent or near-confluent culture. All steps should be performed in a laminar flow hood.

1) Warm PBS, complete medium, and trypsin-EDTA to 37$^{o}$C.
2) Rinse each T25 flask with 5 mL of PBS without calcium and magnesium (10 mL/T75 flask). Swirl gently and remove.
3) Briefly rinse each T25 flask with 3 mL of trypsin-EDTA (6 mL/T75 flask) by pipetting the trypsin onto the bottom of the flask (side opposite of cap). Avoid pipetting the enzyme directly onto the cells. Swirly gently and remove with pipette.
4) Add an additional 1.3 mL of trypsin to each T25 flask (3 mL/T75 flask). Incubate at 37$^{o}$C for 2 minutes.
5) Remove trypsin and add fresh trypsin to each flask (1.3 mL/T25 flask, 3 mL/T75 flask). Rap on flask side 2-3 times, and incubate at 37$^{o}$C for an additional 2 minutes.
6) Visually inspect using contrast-phase microscope to see if cells have detached. If not, rap on flask a few more times. Incubate an additional minute if necessary, and inspect again.
7) To inactivate the enzyme, add 4 mL of complete Claycomb medium to each T25 flask, pipetting directly onto cells.
8) Transfer the cells from the flask to a 15 mL centrifuge tube.
9) Centrifuge cells at 500 rcf for 5 minutes.
10) Meanwhile, remove the gelatin/fibronectin solution from a pre-coated flask. Add 4 mL of complete media to each coated T25 flask. Set aside.

11) Remove the supernatant from the centrifuged cell solution.
12) Gently resuspend the cell pellet in 3 mL of complete Claycomb medium. Pipette up and down to ensure thorough mixing.
13) Add 1 mL of cell solution to the 4 mL of media in the coated flask. Rock flask gently from side to side to distribute cells evenly, and culture at 37$^{o}$C. This is a 1:3 split.
14) If cells are passaged on a Friday, add 2x the amount of media (10 mL/T25 flask instead of 5 mL/T25 flask), and feed the following Monday.

*Freezing cells*

1) Warm PBS, complete medium, trypsin-EDTA, and FBS to 37$^{o}$C.
2) Rinse each T25 flask with 5 mL of PBS without calcium and magnesium (10 mL/T75 flask). Swirl gently and remove.
3) Briefly rinse each T25 flask with 3 mL of trypsin-EDTA (6 mL/T75 flask) by pipetting the trypsin onto the bottom of the flask (side opposite of cap). Avoid pipetting the enzyme directly onto the cells. Swirly gently and remove with pipette.
4) Add an additional 1.3 mL of trypsin to each T25 flask (3 mL/T75 flask). Incubate at 37$^{o}$C for 2 minutes.
5) Remove trypsin and add fresh trypsin to each flask (1.3 mL/T25 flask, 3 mL/T75 flask). Rap on flask side 2-3 times, and incubate at 37$^{o}$C for an additional 2 minutes.
6) Visually inspect using contrast-phase microscope to see if cells have detached. If not, rap on flask a few more times. Incubate an additional minute if necessary, and inspect again.
7) To inactivate the enzyme, add 4 mL of complete Claycomb medium to each T25 flask, pipetting directly onto cells.
8) Transfer the cells from the flask to a 15 mL centrifuge tube.
9) Centrifuge cells at 500 rcf for 5 minutes.
10) Meanwhile, make up cell freezing medium: 95% FBS, 5% DMSO. For the contents of 1 T25 flask, 750-1000 μL is sufficient. For a T75 flask, make up 1.5 mL.
11) Remove the supernatant from the centrifuged cell solution.
12) Gently resuspend the pellet in 1 mL of freezing medium. Immediately transfer contents to a cryovial and freeze at -20$^{o}$C for 45 minutes. Transfer to -80$^{o}$C freezer overnight. Store in liquid nitrogen long-term.

*Thawing cells*

Note: Thaw a cryovial containing one confluent T25 flask into a single coated T25 flask (same goes for T75 flasks).

1) Coat a flask with gelatin/fibronectin. Let sit in incubator overnight at 37$^{o}$C.
2) Prior to thawing cells, remove gelatin/fibronectin solution from the coated T25 flask and add 3 mL of complete Claycomb media to the flask (10 mL/T75 flask). Place back in incubator.
3) Warm complete media to 37$^{o}$C.

4) Transfer 8 mL of complete Claycomb medium into a 15 mL centrifuge tube (10 mL if thawing a T75 flask). Place in water bath.
5) Quickly thaw cells in 37$^{o}$C water bath (~2 minutes) and transfer to the 15 mL centrifuge tube containing complete medium. If necessary, add a small amount of warmed medium to the cryovial to thaw any small residual frozen portion; transfer to the 15 mL conical tube containing complete medium.
6) Centrifuge at 500xg for 5 minutes.
7) Remove the tube from the centrifuge, and remove the supernatant.
8) Gently resuspend the cell pellet in 2 mL of complete Claycomb medium (or 5 mL if thawing T75 flask), and add to the 3 mL (or 10 mL for T75 flask) already in the flask.
9) After cells have attached (~4 hours later), replace the medium with fresh culture medium.

## A.12 Creating a Cell Solution with a Known Concentration Protocol

*Reference:* (Caliari and Harley 2011; Gonnerman, McGregor, et al. in preparation)

*Reagents*

- Complete Claycomb Medium; cover in aluminum foil and store at 4$^{o}$C
    - 87 mL Claycomb Medium (Sigma-Aldrich 51800C ); cover in aluminum foil and store at 4$^{o}$C
    - 10 mL fetal bovine serum (Sigma-Aldrich 12103C, Batch 8A0177, OR Sigma-Aldrich F2442, Batch 058K8426); store at -20$^{o}$C
    - 1 mL pen-strep (Invitrogen 15140-122); store at -20$^{o}$C
    - 1 mL L-glutamine (Invitrogen 25030-081); store at -20$^{o}$C
    - 1 mL Norepinephrine stock solution; store at -20$^{o}$C
      **Safety note: Norepinephrine is highly toxic. Wear appropriate PPE and use a chemical fume hood when weighing out powder.**
        - Make up 100 mL of 30 mM ascorbic acid by adding 0.59 g ascorbic acid (Wako 014-04801) to 100 mL of sterile deionized water.
        - In chemical fume hood, add 80 mg norepinephrine (Sigma-Aldrich A0937) to 25 mL of 30 mM ascorbic acid.
        - Filter sterilize using a 0.22 μm syringe filter (Fisher SLMP025SS)
        - Aliquot in 1 mL volumes and store in sterile microcentrifuge tubes at -20$^{o}$C.
        - Norepinephrine should be made up fresh monthly.

      Note: If there are problems obtaining the medium or FBS, refer to Reservation # 21025944.
- Trypsin-EDTA 0.05% (Invitrogen 25300-062); store at -20$^{o}$C
- Trypan blue (Sigma-Aldrich T8154)
- Sterile phosphate-buffered saline without $Ca^{2+}$ and $Mg^{2+}$ (PBS)

*Supplies and equipment*

- Water bath (37$^{o}$C, Fisher 15-474-35)
- Hemocytometer (Fisher 02-671-5)
- Contrast-phase optical microscope (Leica Microsystems)
- Countess Automated Cell Counter (Invitrogen C10227)
- Countess slides (Invitrogen C10312)
- Sterile serological pipettes (5 mL, 10 mL, 25 mL) (Fisher 13-678-11D, 13-678-11E, 13-678-11)
- Conical centrifuge tubes (15 mL: Fisher 05-527-90, 50 mL: Fisher 14-432-22)

*Procedure*

*Note: All steps should be performed in a laminar flow hood. This procedure describes how to create a cell solution with a concentration of 500,000 cells/scaffold.

1) Warm media, trypsin-EDTA, and PBS to 37$^{o}$C in water bath.

2) Remove media from each flask. Wash each T75 flask with 10 mL of PBS (5 mL/T25 flask). Swirl gently, and remove.
3) Wash each T75 flask with 3 mL of trypsin-EDTA (1.3 mL/T25 flask). Swirl gently, and remove. When passaging cells, do not pipette trypsin directly onto cells; pipette tryspin onto the back of the flask (side opposite the cap).
4) Add 3 mL of fresh trypsin-EDTA to each T75 flask (1.3 mL/T25 flask). Incubate flask at 37$^{o}$C for two minutes.
5) Remove trypsin-EDTA and replace with 3 mL of fresh trypsin-EDTA (1.3 mL/T25 flask). Rap sharply on flask 2-3 times, and incubate flask at 37$^{o}$C for 2-3 minutes.
6) Use microscope to check if cells are detached. Rap on flask a few times to dislodge cells if needed.
7) Deactivate the enzyme by adding 7 mL of complete media to each flask (4 mL/T25 flask). Pipette up and down a couple of times and transfer to a conical centrifuge tube.
8) Pipette contents of centrifuge tube(s) up and down to mix well.
9) Remove a 20 µL sample and add to a PCR tube. Add 40 µL of trypan blue to the tube, and pipette up and down to mix well.
10) Inject 10 µL of cell solution + trypan blue into the hemocytometer.
11) Using the microcope, count the viable cells in each square (5+ squares). Calculate the total cell number using the equation below.
    *Total cell number = (Average number of cells per square) x (Dilution factor*) x (Volume in mL of trypsin-EDTA & complete media) x 10,000.*
    *For a 20 µL of solution mixed with 40 µL of trypan blue, the dilution factor is 3.
12) Inject 10 µL of cell solution + trypan blue into one side of a Countess slide. Under 'settings' change the size gate to 5-30 µm. Ensure that the elongation is set to 80%. Select 'Count' to count the cells. Under 'Details', ensure that the majority of viable cells are circled. Calculate the total cell number using the equation below
    *Total cell number = (Countess' calculated viable cells / mL) x (Correction factor*) x (Volume in mL of trypsin-EDTA & complete media)*
    *The Countess assumes the cell solution:trypan blue ratio is 1:1. Multiply by [dilution factor / 2] to correct.
13) The counts for the hemocytometer and the Countess should be similar (Countess tends to read slightly higher). Recount if necessary. Approximately 4-5 million viable cells are typical for a confluentT25 flask.
14) Centrifuge the cells at 500g for 5 minutes.
15) Remove the media via aspiration/pipetting, taking care not to disturb the cell pellet.
16) Resuspend cell pellet in complete media to get 500,000 cells / 20 µL solution using the following formula:
    *[Volume of media needed] = [Number of cells] / 25 / 1000.*

## A.13 Seeding Cells onto CG Scaffolds Protocol

*Reference:* (Caliari and Harley 2011; Gonnerman, McGregor, et al. in preparation)

*Reagents*

- Complete Claycomb Medium; cover in aluminum foil and store at 4$^{o}$C
    - 87 mL Claycomb Medium (Sigma-Aldrich 51800C ); cover in aluminum foil and store at 4$^{o}$C
    - 10 mL fetal bovine serum (Sigma-Aldrich 12103C, Batch 8A0177, OR Sigma-Aldrich F2442, Batch 058K8426); store at -20$^{o}$C
    - 1 mL pen-strep (Invitrogen 15140-122); store at -20$^{o}$C
    - 1 mL L-glutamine (Invitrogen 25030-081); store at -20$^{o}$C
    - 1 mL Norepinephrine stock solution; store at -20$^{o}$C
      **Safety note: Norepinephrine is highly toxic. Wear appropriate PPE and use a chemical fume hood when weighing out powder.**
        - Make up 100 mL of 30 mM ascorbic acid by adding 0.59 g ascorbic acid (Wako 014-04801) to 100 mL of sterile deionized water.
        - In chemical fume hood, add 80 mg norepinephrine (Sigma-Aldrich A0937) to 25 mL of 30 mM ascorbic acid.
        - Filter sterilize using a 0.22 μm syringe filter (Fisher SLMP025SS)
        - Aliquot in 1 mL volumes and store in sterile microcentrifuge tubes at -20$^{o}$C.
        - Norepinephrine should be made up fresh monthly.

      Note: If there are problems obtaining the medium or FBS, refer to Reservation # 21025944.
- Sterile phosphate-buffered saline without $Ca^{2+}$ and $Mg^{2+}$ (PBS); warmed to 37$^{o}$C
- Hydrated and (optionally) EDAC cross-linked CG scaffolds in PBS

*Supplies and equipment*

- Kimwipes
- Autoclaved round-tip forceps (VWR 82027-394)
- Water bath (37$^{o}$C, Fisher 15-474-35)
- Ultra-low attachment 6-well plates (Fisher 07-200-601)

*Procedure*

*Note: All steps should be performed in a laminar flow hood. This procedure describes how to seed cells at a density 500,000 cells/scaffold.

1) Prior to passaging cells, transfer hydrated scaffolds from PBS to complete Claycomb medium. Let sit in 37$^{o}$C incubator for ~1 hour
2) Passage cells as described in Protocol A.12 to create a cell solution containing 500,000 cells / 20 μL.
3) Transfer scaffolds to ultra-low attachment 6-well plates (3 scaffolds / well).

4) Tip the well plate at an angle so that the media pools at the bottom of each well. Remove the pooled media with a dry Kimwipe.
5) Remove additional moisture by dabbing the scaffolds lightly with a dry Kimwipe. If scaffold curls into a ball, rehydrate in complete media for 10 seconds and repeat dabbing process.
6) Pipette 10 μL of cell solution (5E5 cells/20μL) onto the center of each scaffold.
7) Incubate the scaffolds for 20 minutes at $37^{o}$C.
8) Flip scaffolds over with forceps, and add an additional 10 μL of cell solution to the center of each scaffold.
9) Allow cells to attach for 2 h at $37^{o}$C.
10) Add warmed complete media, covering scaffolds (4-5 mL/well).
11) Change media daily.

### A.14 AlamarBlue Assay for Cell Metabolic Activity Protocol

*Reference:* (Tierney, Jaasma et al. 2009; Caliari and Harley 2011; Gonnerman, McGregor et al. in preparation; Gonnerman, Turgeon, et al. in preparation)

*Reagents*

- Complete Claycomb Medium; cover in aluminum foil and store at 4°C
    - 87 mL Claycomb Medium (Sigma-Aldrich 51800C ); cover in aluminum foil and store at 4°C
    - 10 mL fetal bovine serum (Sigma-Aldrich 12103C, Batch 8A0177, OR Sigma-Aldrich F2442, Batch 058K8426); store at -20°C
    - 1 mL pen-strep (Invitrogen 15140-122); store at -20°C
    - 1 mL L-glutamine (Invitrogen 25030-081); store at -20°C
    - 1 mL Norepinephrine stock solution; store at -20°C
      **Safety note: Norepinephrine is highly toxic. Wear appropriate PPE and use a chemical fume hood when weighing out powder.**
        - Make up 100 mL of 30 mM ascorbic acid by adding 0.59 g ascorbic acid (Wako 014-04801) to 100 mL of sterile deionized water.
        - In chemical fume hood, add 80 mg norepinephrine (Sigma-Aldrich A0937) to 25 mL of 30 mM ascorbic acid.
        - Filter sterilize using a 0.22 μm syringe filter (Fisher SLMP025SS)
        - Aliquot in 1 mL volumes and store in sterile microcentrifuge tubes at -20°C.
        - Norepinephrine should be made up fresh monthly.

      Note: If there are problems obtaining the medium or FBS, refer to Reservation # 21025944.
- AlamarBlue (Invitrogen DAL1100); store at 4°C
- Sterile phosphate-buffered saline without $Ca^{2+}$ and $Mg^{2+}$ (PBS)

*Supplies and equipment*

- 24-well plates (Fisher 08-772-1)
- 96-well plates (Fisher 12-565-66)
- MTS 2/4 digital microtiter shaker (IKA 3208001)
- Water bath (37°C, Fisher 15-474-35)
- Fluorescent spectrophotometer (Tecan F200)
- Autoclaved round-tip forceps (VWR 82027-394)

*Procedure*

*Note: All steps should be performed in a laminar flow hood. Volumes chosen are good for scaffolds 6-8 mm in diameter, 3 mm in height, with 500,000 cells/scaffold seeding density (assuming 300% maximum attachment). Adjust volumes and incubation times for different size scaffolds/seeding densities as needed.

*AlamarBlue Standard*

*Note: Reserve a portion of the cell-seeding solution to create a new standard for each experiment, as metabolic activity can vary from passage to passage.

1) Warm complete media and alamarBlue to 37°C.
2) Passage cells as described in Protocol A.12 to create a solution with a concentration of 5E5 cells/20 µL.
3) Dilute the above solution to form a solution with a concentration of 2E4 cells/10 µL by combining 2875 µL of complete media with 250 µL of 5E5/20 µL cell solution.
4) To a 24-well plate, add the following volumes (in µL) of media, 2E4/10 µL cell solution, and alamarBlue:

| | *Well 1* | *Well 2* | *Well 3* | *Well 4* | *Well 5* | *Well 6* | *Well 7* | *Well 8* |
|---|---|---|---|---|---|---|---|---|
| | ***Media blank*** | ***AB & media blank*** | ***2.5E5 cells*** | ***5E5 cells*** | ***7.5E5 cells*** | ***1E6 cells*** | ***1.25E6 cells*** | ***1.5E6 cells*** |
| **Media** | 1000 | 900 | 775 | 650 | 525 | 400 | 275 | 150 |
| **2E4/10µL cell solution** | 0 | 0 | 125 | 250 | 375 | 500 | 625 | 750 |
| **AlamarBlue** | 0 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |

5) Incubate under moderate shaking at 37°C for 50 minutes. Check frequently, as this time can vary.
   Note: During this time, the alamarBlue will be converted to alamarRed, and the solution will progressively become more and more purple. If the incubation period is chosen is too long, the intensities for the highest cell number data points will be the same, and the standard curve will plateau.
6) From each well, pipette 100 µL in triplicate into a 96-well plate.
   Immediately read plate on Tecan F200 fluorometer. Load protocol: "alamarBlue F200 Emily". Excitation: 352 nm, Emission: 461 nm, Gain: 37.
   Plot normalized intensity (y axis) vs. cell number (x axis).

$$[Normalized\ intensity] = [Average\ sample\ intensity] - [AB\ \&\ media\ blank\ intensity]$$

   Create a best-fit linear curve.

*AlamarBlue Assay*

1) Warm complete media, PBS, and alamarBlue to 37$^{o}$C.
2) Pipette 1000 µL of media into the first well of a 24-well plate. Pipette 1000 µL of 10% alamarBlue solution into the remaining wells. Use the following table as a guideline:

| | *Well 1* | *Well 2* | *Wells 3,4,5…* |
|---|---|---|---|
| | ***Media blank*** | ***AB & media blank*** | ***Scaffolds*** |
| **Media** | 1000 µL | 900 µL | 900 µL |
| **AlamarBlue** | 0 µL | 100 µL | 100 µL |

3) Briefly dip each scaffold in PBS, and place in the appropriate well.
4) Incubate under moderate shaking at 37$^{o}$C for the time determined by the standard (~1 h).
5) From each well, pipette 100 µL in triplicate into a 96-well plate.
   Immediately read plate using the Tecan F200 fluorometer. Load protocol: "alamarBlue F200 Emily". Excitation: 352 nm, Emission: 461 nm, Gain: 37.
   Convert the average normalized intensity into an equivalent cell number for each scaffold using the standard curve.

## A.15 Hoechst DNA Quantification Protocol

*Reference:* (Kim, Sah et al. 1988; Caliari and Harley 2011; Gonnerman, McGregor, et al. in preparation; Gonnerman, Turgeon et al. in preparation)

*Reagents*

- Hoechst dye buffer (500 mL); store at 4ºC for up to 3 months
    - 450 mL deionized water
    - 5.84 g sodium chloride (0.1 M)
    - 0.605 g Tris base
    - 0.185 g disodium EDTA (Sigma-Aldrich E5134)

  Add reagents to large beaker with stir bar. Bring total volume to 500 mL by adding additional DI water. Adjust pH to 7.4 by adding concentrated HCl/NaOH drop-wise. Sterile filter before use.
- Papain buffer (100 mL); store at 4ºC
    - 100 mL PBS
    - 1 mL 0.5 M EDTA (pH = 8.0, Sigma-Aldrich EDS); store at 4ºC
    - 79 mg cysteine-HCl (Sigma-Aldrich 00320)
- Hoechst 33258 dye solution (1 mL); store at 4ºC for up to 6 months
    - 1 mL sterile water
    - 1 mg Hoechst 33258 dye (Invitrogen H1398); store at 4ºC
- Papain from Carica papaya (Sigma-Aldrich 76218); store at -20ºC

*Supplies and equipment*

- Black 96-well plates (Fisher 14-245-177)
- Vortex (Fisher 02-215-365)
- Water bath (60ºC, Fisher 15-460-2SQ)
- Fluorescent spectrophotometer (Tecan F200)
- Microcentrifuge tubes (1.5 mL)
- Conical centrifuge tubes (15 and 50 mL)
- Kimwipes
- Sterile blunt-nosed tweezers

*Procedure*

Note: New standard curves should be generated at least once every 1-2 months. The volumes used in the procedure below are designed for the analysis of a 6 mm diameter scaffold disc, 3 mm thick, seeded with 500,000 cells (measuring up to 300% attachment).

*DNA Standard*

1) Prior to passaging cells, make up papain buffer solution. Add 2.4 mg of papain to 1 mL of papain buffer. Let solubilize in 60ºC water bath for ~10 minutes. Vortex thoroughly to mix.

2) In laminar flow hood, make up 400 µL solution containing 150,000 cells/30 µL papain digest (i.e. 2 million cells/400 µL). Let digest in 60°C water bath for 24 hours.
3) Vortex Hoechst dye solution, and add 2 µL of Hoechst dye solution to 10 mL of Hoechst dye buffer to create 'dye/buffer.' Vortex to mix.
4) Label microcentrifuge tubes with 'B', '2', '4', '6', … '28', '30' (for amount of cell digest to be added, in µL).
5) Add 600 µL of dye/buffer to labeled microcentrifuge tubes.
6) To each tube, add the following volumes of papain buffer and cell digest:

| | **B** | **2** | **4** | **6** | **8** | **10** | **12** | **14** | **16** | **18** | **20** | **22** | **24** | **26** | **28** | **30** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Papain buffer (µL)** | 30 | 28 | 26 | 24 | 22 | 20 | 18 | 16 | 14 | 12 | 10 | 8 | 6 | 4 | 2 | 0 |
| **Cell digest (µL)** | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 | 30 |

Each tube should receive 30 µL total of papain buffer/cell digest, which will bring the total volume in each tube to 630 µL.

7) Vortex each tube thoroughly.
8) Pipette 190 µL in triplicate from each tube into a black 96-well plate.
9) Immediately read plate on Tecan F200 fluorometer. Load protocol: "DNA F200". Excitation: 352 nm, Emission: 461 nm, Gain: 46.
10) Plot normalized intensity (y axis) vs. cell number (x axis).

$$[Normalized\ intensity] = [Average\ sample\ intensity] - [Blank\ intensity]$$

$$[Number\ of\ cells] = \frac{[Cell\ digest\ volume, \mu L]}{30\ \mu L} * 150{,}000 * \frac{300\ \mu L}{30\ \mu L}$$

Create a best-fit linear curve.

*Analyzing Samples – Day 1*

1) Make up papain buffer solution. Add 21.6 mg of papain to 9 mL of papain buffer (2.4 mg/mL). Let solublize in hot water bath for ~10 minutes. Vortex thoroughly to mix.
2) Label microcentrifuge tubes with sample names, including one scaffold blank (scaffold with no cells), and one blank (no scaffold).
3) In laminar flow hood, lightly blot each scaffold with a Kimwipe to remove excess media. Add one scaffold to each tube; tap the base to ensure scaffold will be submerged in papain digest.
4) Pipette 300 µL of the papain digest solution into labeled microcentrifuge tubes.
5) Incubate in water bath at 60°C for 24 hours.

*Analyzing Samples – Day 2*

Label microcentrifuge tubes (one for each sample plus two for blanks).

1) Add 2 µL of vortexed Hoechst dye solution to 10 mL of Hoechst dye buffer.
2) Vortex dye/buffer thoroughly.
3) Pipette 600 µL of dye/buffer solution into each tube.
4) Vortex digested scaffolds thoroughly.
5) Pipette 30 µL of vortexed digest from each microcentrifuge tube into its corresponding tube of 600 µL dye/buffer.
6) Vortex buffer/dye + digest thoroughly.
7) Pipette 190 µL in triplicate from each tube into a black 96-well plate.
8) Immediately read plate on Tecan F200 fluorometer. Load protocol: "DNA F200". Excitation: 352 nm, Emission: 461 nm; Gain: 46.
9) Normalize intensity by subtracting the fluorescent intensity of the scaffold blank from the average fluorescent intensity of each sample. Using the linear best-fit equation generated from the standard curve, convert the normalized intensity into a cell number.

## A.16 Determining the Presence/Absence of HL-1 Cell Beating in CG Scaffolds Protocol

*Reference:* (Gonnerman, McGregor, et al. in preparation)

*Reagents*

- HL-1 cells seeded onto CG scaffolds, fully submerged in complete Claycomb medium

*Supplies and equipment*

- Ultra-low attachment 6-well plates
- Contrast-phase optical microscope with camera (Leica Microsystems DMIL LED with DFC295 camera)
- Video imaging software (MultiTime Module for Leica Application Suite)

*Procedure*

* Note: It is best to be alone in the room when capturing videos, as slight disturbances (i.e. people walking past the microscope) will be picked up by the camera and could falsely be interpreted as beating.

1) Position the scaffold so that it is centered over the light source by gently rocking the 6-well plate. It is easiest to spot beating if the scaffold is oriented as an upright cylinder and not on its side.
2) Change from looking through the microscope eyepiece to looking at the computer screen (as beating is much easier to detect on the computer screen).
3) Turn the microscope objective to 20x, and set the filter to either 20x or 40x. Adjust the brightness so that the scaffold struts are just visible near the scaffold perimeter. Adjust the focus to maximize the struts visualized.
4) A beating scaffold is defined as a scaffold that contains a region with struts pulsating in a regular interval.
5) Look at n >6 distinct regions of the scaffold prior to labeling that scaffold as ‘center beating,’ ‘perimeter beating,’ or ‘no beating’ depending on the presence/absence of beating and if applicable, the beating location.
6) Take representative videos using the ‘capture’ function in LAS MultiTime Movie. Note that the default compression setting results in very large files.

## A.17 Tagging Cells with CellTracker Green Fluorescent Dye Protocol

*Reference:* (Gonnerman, Turgeon, et al. in preparation)

*Reagents*

- CellTracker Green CMFDA; store at -20°C (Invitrogen C7025)
- Complete Claycomb Medium; cover in aluminum foil and store at 4°C
    - 87 mL Claycomb Medium (Sigma-Aldrich 51800C ); cover in aluminum foil and store at 4°C
    - 10 mL fetal bovine serum (Sigma-Aldrich 12103C, Batch 8A0177, OR Sigma-Aldrich F2442, Batch 058K8426); store at -20°C
    - 1 mL pen-strep (Invitrogen 15140-122); store at -20°C
    - 1 mL L-glutamine (Invitrogen 25030-081); store at -20°C
    - 1 mL Norepinephrine stock solution; store at -20°C
      **Safety note: Norepinephrine is highly toxic. Wear appropriate PPE and use a chemical fume hood when weighing out powder.**
        - Make up 100 mL of 30 mM ascorbic acid by adding 0.59 g ascorbic acid (Wako 014-04801) to 100 mL of sterile deionized water.
        - In chemical fume hood, add 80 mg norepinephrine (Sigma-Aldrich A0937) to 25 mL of 30 mM ascorbic acid.
        - Filter sterilize using a 0.22 μm syringe filter (Fisher SLMP025SS)
        - Aliquot in 1 mL volumes and store in sterile microcentrifuge tubes at -20°C.
        - Norepinephrine should be made up fresh monthly.

      Note: If there are problems obtaining the medium or FBS, refer to Reservation # 21025944.
- Dimethyl sulfoxide (DMSO)
- Phosphate buffered saline without $Ca^{2+}$ and $Mg^{2+}$ (PBS)

*Supplies and equipment*

- Conical centrifuge tubes (15 mL: Fisher 05-527-90)
- Sterile serological pipettes (5 mL: Fisher 13-678-11D)
- Micropipettes and tips
- Tecan M200 fluorometer

*Procedure*

*This procedure describes how to stain a single confluent T25 flask of cells. Scale volumes accordingly.

1) Mix the as-available tube of CellTracker Green CMFDA dye with 11 μL of DMSO.
2) Add 5 mL of complete media to conical tube.
3) Add 5 μL of dye-DMSO to the 5 mL of complete media. Mix well.
4) Remove media from culture flask. Add media containing dye-DMSO to culture flask.
5) Incubate at 37°C for 20 minutes.
6) Remove media from culture flask.

7) Rinse twice with PBS.
8) Passage cells normally as described in Protocol A.12.
9) Seed the desired number of cells onto individual scaffolds or scaffolds within an array as described in Protocol A.13 with the following exceptions: Do not place the scaffolds in media prior to seeding; resuspend the cell pellet in PBS instead of media; seed the cells on one side of the scaffold only (do not flip the scaffolds or array upside-down); use 96-well plates for seeding, rather than ultra-low attachment 6-well plates.
10) Cells seeded directly onto 96-well plates in 100 μL of PBS serve as controls.
11) Place the 96-well plate dimensioned scaffold array on a clear 96-well plate lid so that the scaffolds are directly above the well circles.
12) Quantify fluorescent intensity using a Tecan M200 fluorometer with multiple (e.g. 24) reads per well. Excitation: 492 nm (BP 9 nm); Emission: 517 nm (BP 20 nm); Gain: 78.

## A.18 Cell Orientation Analysis for Cells Fixed within CG Scaffolds Protocol

*Reference:* (Caliari and Harley 2011; Gonnerman, McGregor et al. 2011)

*Reagents*

- Formalin solution, 10% formaldehyde in neutral buffer(Polysciences 08379-3.75)
- H&E stain

*Supplies and equipment*

- Leica Contrast-Phase Microscope
- ImageJ Software
- Microsoft Excel

*Procedure*

*This procedure requires the use of the "OrientationJ" plugin for ImageJ. It can be downloaded from the following website: http://bigwww.epfl.ch/demo/orientation/ under "Installation for final users."

1) Fix scaffolds in formalin solution in labeled microcentrifuge tubes. Store at 4$^{o}$C overnight.
2) Place scaffolds in embedding cartridges, noting the orientation of each. For longitudinal samples, it may be easiest to cut the scaffold in half using a razor blade prior to placing it into the cartridge so that the scaffold can stand upright.
3) Samples are run through a processor and embedded in paraffin wax. When cutting, save every tenth section (~50-75 μm). Save 15 sections for each block, doing H&E stains on slides 1, 8, 15. Unstained slides can later be stained for cardiac markers.
4) Using a contrast-phase microscope on the open filter (to get the maximum color contrast for the H&E stain, with cells appearing purple and scaffold pink), capture ~3 images per slide (9 per sample) for a total of 400-500 cells per sample. A magnification of 20x appears to work best for HL-1 cells. Save images as TIF files.
5) Use the OrientationJ plugin for ImageJ to analyze each cell's orientation angle:
   File > Open [filename.tif].
   Image > Color > Channels Tool.
   Select "Color" from dropdown menu.
   Select the "OK" button.
   Plugins > OrientationJ_ > OrientationJ Measure.
   Highlight the cell to analyze using the oval drawing tool.
   Press the "Measure" button.
   Note: Make sure that the oval drawn encapsulates the entire cell.
6) Analyzing each cell will give three orientation angles (one for each color channel), but the values should be similar. Copy and paste data into Excel.
7) Create a histogram of the data using Excel.

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