Tunable substrate with electrodes for cardiomyocyte maturation compatible with high throughput testing

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Honors Capstone Report

Introduction

Human induced pluripotent stem cells (hiPSCs) have the potential to differentiate into any type of somatic cell. Applications of *in vitro* culturing using hiPSCs has allowed for research in cardiac and neuroscience fields to occur without invasive procedures.[1] Pluripotent stem cells are already being used to discover and test new drugs and model diseases.[2] Furthermore, induced pluripotent stem cell-derived cardiomyocytes(iPSC-CMs) from patient lines provide the capability for patient-specific testing but have been limited by the lack of a mature phenotype.[3]

When developing new drugs, cardiotoxicity needs to be tested. Each year, the pharmaceutical industry invests billions of dollars into drug development to develop effective medications with minimal side effects. Of the drugs that pass safety screening, some of them are later removed from the market due to resulting cardiovascular complications. Existing technologies for cardiotoxicity screening fall short of modeling the complex nature of the human heart. Induced pluripotent stem cell-derived cardiomyocytes capture aspects of the heart that current methodologies are lacking. *In vitro* testing using iPSC-CMs has the potential to provide insight to patient and population specific screening.[4]

Existing structures designed to culture cardiomyocytes provide physical stimulation and a platform to study the effects of electrical stimulus.[3] The process of creating these structures requires the fabrication of molds to cast the pillars and additional molds for support structures. Creating these multi-part platforms to mature tissues is a complicated manufacturing process that is not viable for high throughput testing. Tools such as the biowire (Nunes et al.) do not include physical stimulation which can further encourage an adult phenotype.[5]

Kirigami is the traditional Japanese art of paper cutting through which paper takes a three dimensional form. These concepts and ideas are applied to inspire the design of an easily manufacturable substrate compatible with high throughput testing and a custom well plate. Additionally, gold electrodes can be deposited on the substrate to provide electrical pacing. The resulting scaffold is an easily manufacturable, tunable, substrate that provides mechanical and electrical stimulus for culturing cardiomyocytes *in vitro.*

Background

The immaturity of iPSC-CMs *in vitro* is a limitation. Studies have shown that physical and electrical stimulation aid in maturation of iPSC-CMs. [3, 5] Addressing the lack of a mature phenotype in these tissues observed for *in vitro* models, Nunes et al. created biowires to mature tissues with electrical stimulation. The tissues form around surgical sutures and then are exposed to: no electrical stimulation, low-frequency ramp up regimen, or a high-frequency ramp up regimen. Both ramp up regimens increase using the same increment of 1.83 Hz. It was found that the high-frequency ramp up rate resulted in tissues with a more adult phenotype when compared to samples without stimulation or a frequency ramp up rate that did not surpass 3 Hz.[5] *In vivo* cardiac tissues beat more slowly after birth but studies have shown that higher stimulation frequencies are more beneficial for achieving an adult phenotype *in vitro*.[5] The iPSCs are passaged before inducing cardiac differentiation. After 12 days the cells are characterized and result in 80-90% of cells with the marker cTnT that is specific to cardiomyocytes. These cells are

then used in experiments as is without further cardiomyocyte selection.[3] Furthermore, responsiveness of hiPSC-CMs decreases throughout differentiation.[3] Ronaldson-Bouchard et al. tested the effect of beginning the electrical stimulation regimen earlier on, while there is high cell plasticity. Human induced pluripotent stem cell-derived cardiomyocytes were tested at an early stage(day 12 once spontaneous contractions begin) and at a later stage(day 28 in culture).[3] Results indicate that adult-like cardiac cells can be grown in four weeks from differentiated early stage iPSCs *in vitro* when suspended in a fibrin hydrogel and subjected to increasing electromechanical stimulation. The mechanical loading is intended to model the mechanical loading during fetal postnatal transition. It is the implementation of electrical stimulation during high cell plasticity as an increasing regimen that has led to a more adult-like phenotype for *in vitro* cardiac tissues.[3] It was also found that although the molecular, structural, and metabolic features matured quickly, there was an inverse relationship with the establishment of mature cardiac function. It is thought that four weeks may not be long enough for tissues to establish features observed in adult myocardium.[3]

The Food and Drug Administration launched the Comprehensive *in vitro* Proarrhythmia Assay (CiPA) initiative in 2013 with the goal of better understanding which drugs cause torsades de pointes (TdP).[6] Torsades de pointes is a type of tachycardia that is characterized by changes in amplitude of the QRS complex on an electrocardiogram.[7] Patients with TdP may experience syncope, hypotension, and/or a rapid pulse.[7] Left untreated, TdP can be fatal. However, little is known about drug-induced TdP as the medications that have been correlated with TdP have varying structures.[6, 7] In response to medications on the market that caused TdP, the FDA along with European and Japanese regulatory agencies pulled those drugs from shelves. Additionally, they developed regulatory guidelines for future drug development. These guidelines recommended testing the inhibitory effect of the drug on the hERG protein, a voltage-gated potassium channel found in the heart that controls electrical activity. The agencies found that when the hERG protein was affected, there was an association with the development of TdP. Lastly, testing the effect of the drug on the length of the QT interval was recommended. With these guidelines in place, no new drugs on the market caused TdP. However, further research found that not all proposed drugs that interacted with the hERG protein lead to the development of TdP. This negatively impacted the progress of pharmaceutical development as medications with promising results did not reach the market. From this, the CiPA initiative was created to better evaluate the relationship between pharmacological agents and torsades de pointes.[6] The initiative strives to standardize the *in vitro* assays used for characterizing drug effects, standardize *in silico* models, and set the standard for best practices for stem cell derived cardiomyocyte modeling.[8]

Concept and design

Inspired by origami and kirigami, the substrate is formed from a sheet of 50 micron thick biaxially oriented polyethylene terephthalate (PET). Using CAD to design the pattern (Figure 1A), it can be easily customized and optimized for specific applications. As shown in Figure 1A, the pattern includes small holes at the ends of the pillars to prevent the tissues from falling off the pillars. An extension on the base of the substrate allows for easy access to electrodes intended for use with alligator clips when integrated with electrical pacing. Shown in yellow is the electrode pattern that can be deposited to integrate electrical pacing (Figure 1A). Creating the

substrate from a sheet of PET eliminates the need for casting. Once laser cut, the PET is thermoformed on an aluminum form (Figure 3) to create a three dimensional substrate (Figure 1C). The mold has an accompanying topper to sandwich the substrate. Two of these thermoformed sheets are layered antiparallel with 5mm between each pair of pillars (Figure 1D and E). The substrate is designed to have 5mm between each pair of pillars to form tissues with a two to one aspect ratio.

A custom well plate is cast from PDMS featuring a glass bottom for clear imaging of tissues (Figure 1F). The mold for the well plate is 3D printed. Another design element of the well plate is a trough system along the perimeter of the plate. This trough is intended for phosphate buffered saline (PBS) to maintain the humidity of the culture. The rectangular wells are optimized for the substrate to culture tissues with a two to one aspect ratio.

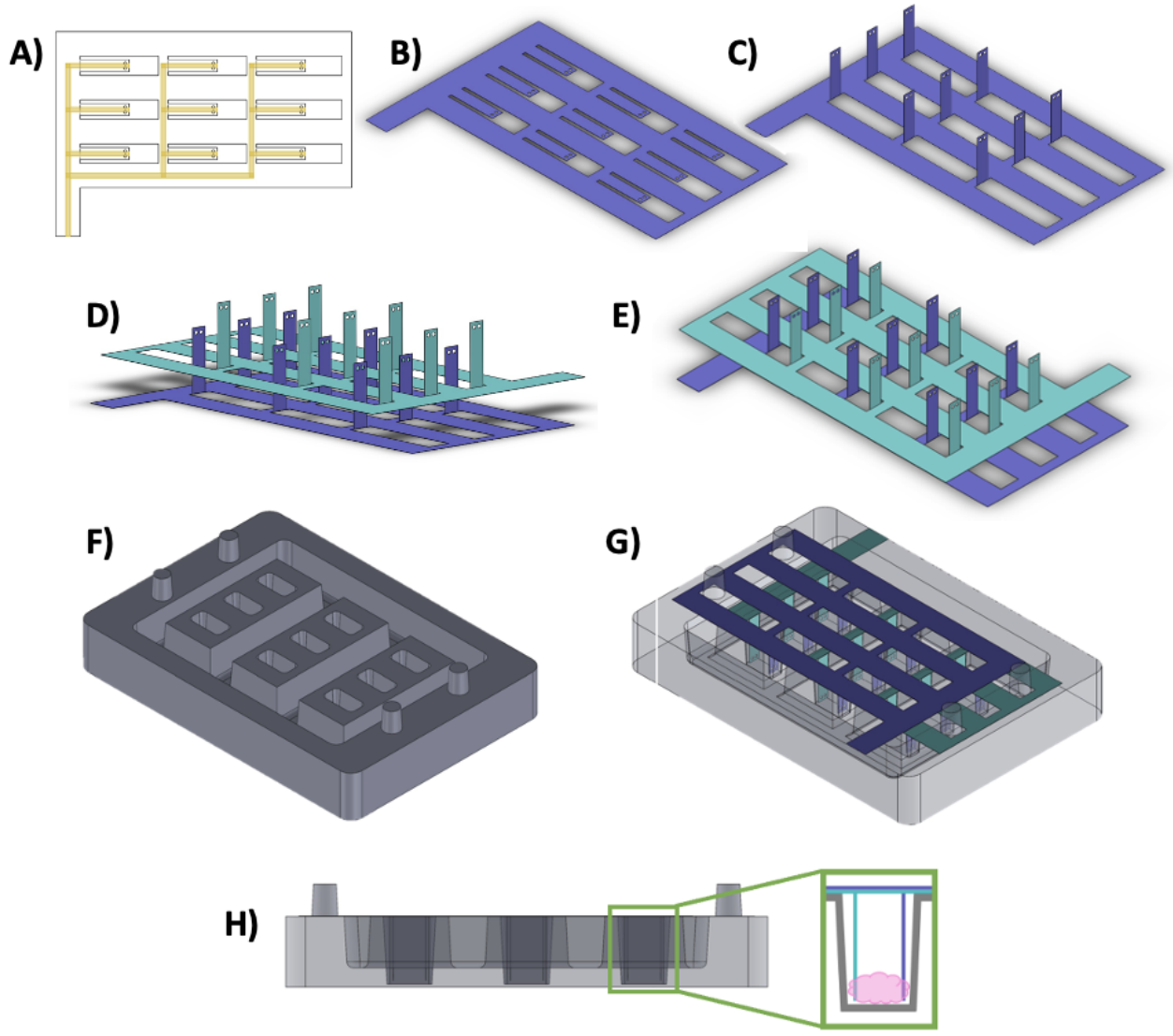

Figure 1. A) A unit cell of the kirigami substrate pattern. Gold lines show optional electrode patten. B) Single layer substrate. C) Thermoformed substrate layer. D) Substrates are layered antiparallel to form a scaffold with a pair of pillars for each well. E) Complete kirigami substrate. F) Custom well plate G) Kirigami substrate inverted over well plate. H) Side view of substrate and well system.

Methods

A variety of materials are needed to create the kirigami scaffold. The following section summarizes the materials and methods to fabricate these substrates.

Laser Cutting

Substrate patterns are laser cut from 50um thick biaxially oriented PET (Figure 1A). If electrodes are needed, a mask is cut from 100µm PET (Figure 2).

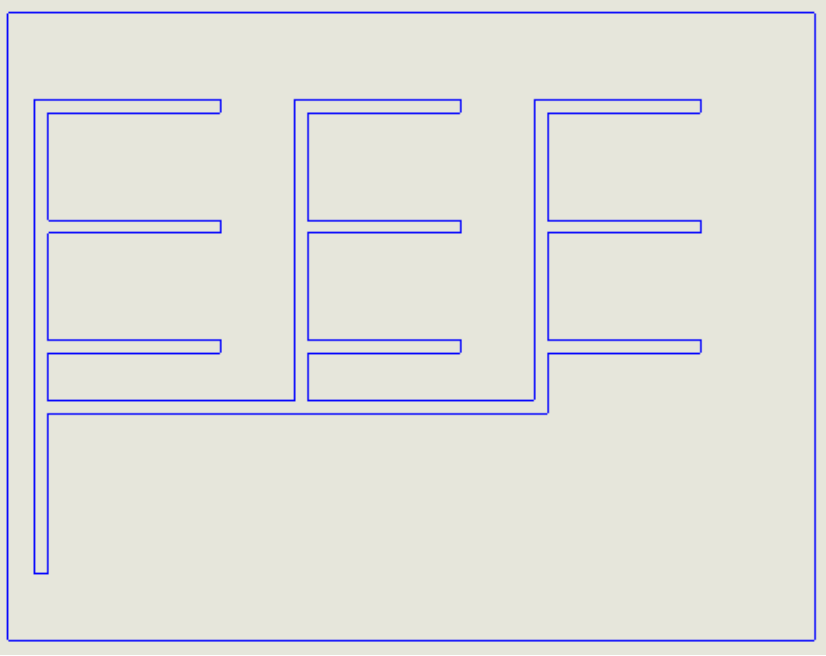

Figure 2. Mask pattern for electrodes.

Cleaning

The substrates and masks are cleaned using a series of sonication baths. First, sonicate in a soapy water bath for 15 minutes at 40°C. This is followed by baths of deionized water, acetone, and isopropyl alcohol, each for 15 minutes at 40°C. The last step is to heat additional isopropyl alcohol to 185°C before placing the substrate in the bath and then continuing to heat until 215°C. Once 215°C is reached, the substrates are left in the bath for three minutes. Dry the substrates using nitrogen.

Depositing Electrodes (optional)

A mask is used for the electrode patterning in a thermal evaporation chamber (Figure 2). The electrode consists of a 5nm thick chromium adhesion layer and a 50 nm gold layer.

Thermoforming

To thermoform the substrate, pre-heat the furnace to 145˚C. Then place the laser cut substrate onto the puncher and tape it down so that the pillars of the substrate are parallel with and resting on the pillars of the puncher. Next, place the topper on the puncher, ensuring that no pillars are trapped between the puncher and the topper. Clamp the puncher and topper together and then clamp pillars of substrate to the puncher and place in the furnace for 30 minutes. Allow to cool to room temperature before removing the substrate.

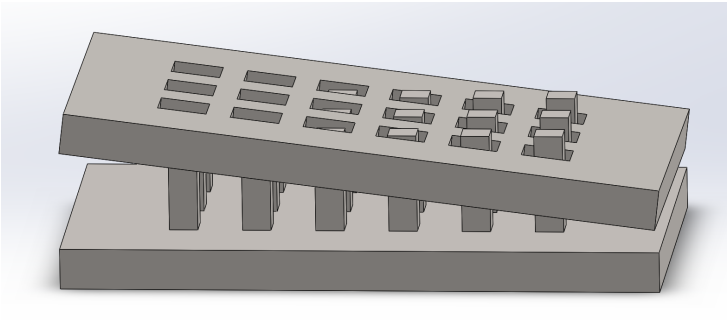

Figure 3. A puncher and topper were machined from aluminum for thermoforming.

Assembly

Two thermoformed sheets are glued together using epoxy to create a substrate with a pair of pillars for each corresponding well. One thermoformed sheet is placed on a 3D printed assembly guide with the pillars flush against the blocks on the assembly guide (Figure 4). Before placing the second sheet, apply epoxy then quickly align the second layer such that the pillars are flush against the opposite of the block. Ensure the layers are adhering by applying pressure where the epoxy was deposited. Once the epoxy has set, remove the scaffold from the assembly guide (Figure 5).

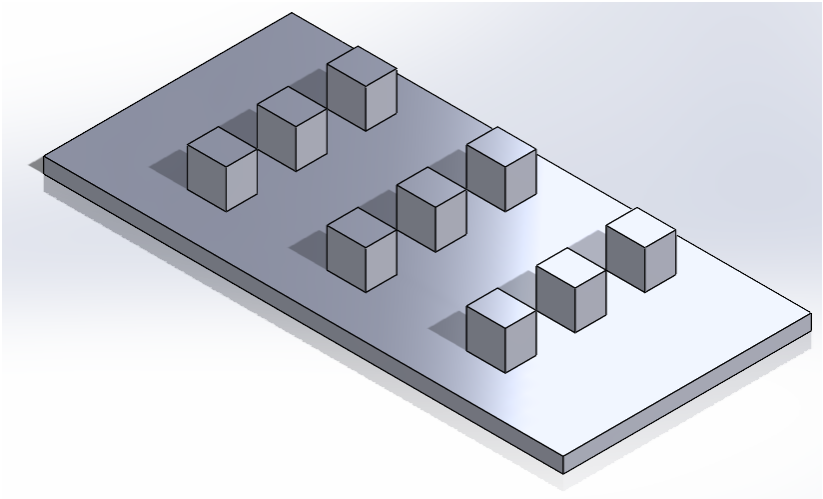

Figure 4. Assembly guide with nine blocks to ensure pillars are spaced 5mm apart.

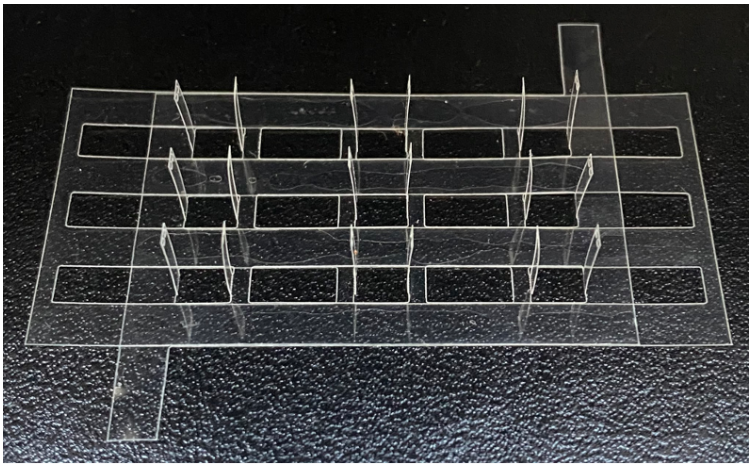

Figure 5. Kirigami scaffold.

Properties

The kirigami scaffold is designed to be easily tunable. This section summarizes mechanical properties and additional features of the substrate.

Mechanical

The mechanical properties of the structure can be tuned through material selection and substrate design. The thickness of the sheet and width of the pillar affect the bending stiffness of the pillars. Bending stiffness is measured by deflecting both pillars and can be optimized for specific applications. Figure 6A shows the change in bending stiffness between the two pillars for different geometries. A micromanipulator and MATLAB were used to measure the deflection and calculate the bending stiffness (Appendix I). As *in vitro* cell cultures of cardiomyocytes take several weeks to mature and subject scaffolds to cyclic loading, it is crucial that there is not a decline in the mechanical properties of the substrate (Figure 6B). Using a micromanipulator, the pillars were tested at 2Hz showing no significant correlation between time of cyclic loading and displacement.

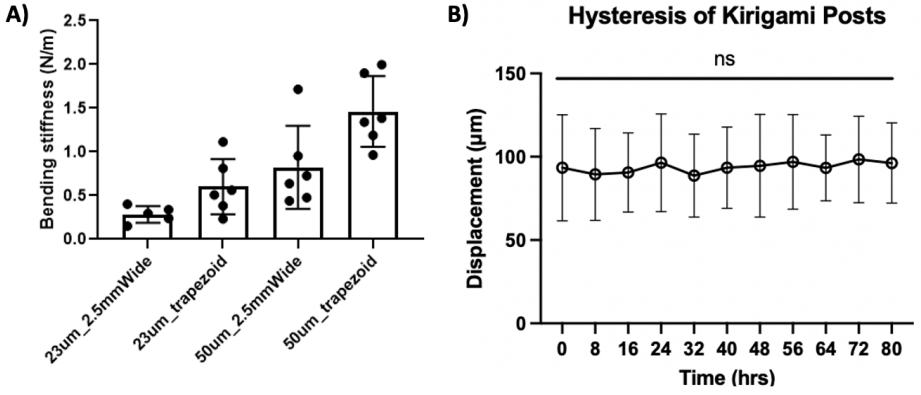

Figure 6. A) Graph showing the measured bending stiffness for different pillar geometries. B) Hysteresis testing of pillars indicating no significant differences over 80 hours. No significant correlation between time of cyclic loading and displacement using one-way ANOVA was found ($p>0.05$).

The tissues compact on the pillars and the contractile forces stress the pillars. Using Abaqus, the stresses generated by these forces can be visualized (Figure 7). The pillars were each loaded with 1mN of force toward each other, representative of the force observed in early stage intensity trained tissues by Ronaldson-Bouchard et al.[3]

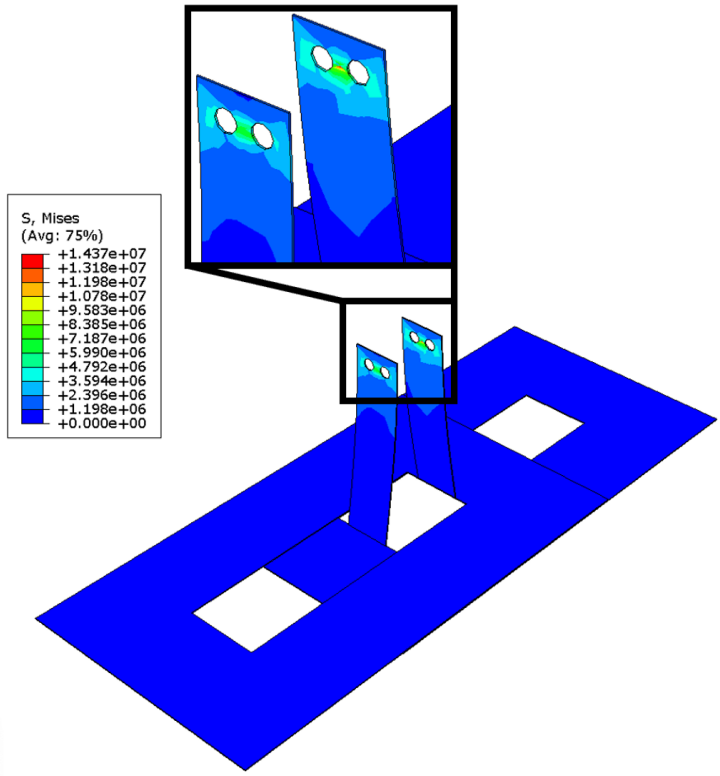

Figure 7. Mechanical modeling of force applied at the tips of the pillars using Abaqus.

Cyto-compatible/non-compatible

The PET and Cr/Au electrodes are biocompatible which allow for tissues to form and mature on the pillars.

Additional features of tool

The holes at the ends of the pillars aid in tissue placement. These provide “holds” for the tissues to prevent them from falling off the pillars. Furthermore, electrodes can be added if electro-pacing is required. The design includes a handle extension on the base of the substrate to provide an easily accessible contact point for alligator clips.

Conclusion

The kirigami substrate and rectangular well plate provide sufficient conditions for tissues to mature while providing mechanical stimulation. Further, this design can be manufactured rapidly, and optimized for specific applications. This results in a scaffold that is compatible with high throughput testing. Gold electrodes can be deposited to provide electrical stimulation. Further work will focus on the integration of a pacing system.

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Appendices

Appendix I. Bending stiffness calculations

$$bending\ stiffness\ =\ \frac{F}{\delta}$$

$$F\ =\ \frac{3dEI}{L^3}$$

$$I\ =\ \frac{\pi r^4}{4}$$

Where:

$d\ =\ rod\ deflection$

$E\ =\ rod\ elastic\ modulus$

$L\ =\ length\ of\ rod$

$r\ =\ radius\ of\ rod$

$\delta\ =\ post\ deflection$