Video Article

Capillary Force Lithography for Cardiac Tissue Engineering

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Abstract

Cardiovascular disease remains the leading cause of death worldwide[1]. Cardiac tissue engineering holds much promise to deliver groundbreaking medical discoveries with the aims of developing functional tissues for cardiac regeneration as well as *in vitro* screening assays. However, the ability to create high-fidelity models of heart tissue has proven difficult. The heart's extracellular matrix (ECM) is a complex structure consisting of both biochemical and biomechanical signals ranging from the micro- to the nanometer scale[2]. Local mechanical loading conditions and cell-ECM interactions have recently been recognized as vital components in cardiac tissue engineering[3-5].

A large portion of the cardiac ECM is composed of aligned collagen fibers with nano-scale diameters that significantly influences tissue architecture and electromechanical coupling[2]. Unfortunately, few methods have been able to mimic the organization of ECM fibers down to the nanometer scale. Recent advancements in nanofabrication techniques, however, have enabled the design and fabrication of scalable scaffolds that mimic the *in vivo* structural and substrate stiffness cues of the ECM in the heart[6-9].

Here we present the development of two reproducible, cost-effective, and scalable nanopatterning processes for the functional alignment of cardiac cells using the biocompatible polymer poly(lactide-co-glycolide) (PLGA)[8] and a polyurethane (PU) based polymer. These anisotropically nanofabricated substrata (ANFS) mimic the underlying ECM of well-organized, aligned tissues and can be used to investigate the role of nanotopography on cell morphology and function[10-14].

Using a nanopatterned (NP) silicon master as a template, a polyurethane acrylate (PUA) mold is fabricated. This PUA mold is then used to pattern the PU or PLGA hydrogel via UV-assisted or solvent-mediated capillary force lithography (CFL), respectively[15,16]. Briefly, PU or PLGA pre-polymer is drop dispensed onto a glass coverslip and the PUA mold is placed on top. For UV-assisted CFL, the PU is then exposed to UV radiation (λ = 250-400 nm) for curing. For solvent-mediated CFL, the PLGA is embossed using heat (120 °C) and pressure (100 kPa). After curing, the PUA mold is peeled off, leaving behind an ANFS for cell culture. Primary cells, such as neonatal rat ventricular myocytes, as well as human pluripotent stem cell-derived cardiomyocytes, can be maintained on the ANFS[2].

Video Link

The video component of this article can be found at http://www.jove.com/video/50039/

Introduction

Cardiovascular disease is the leading cause of morbidity and mortality in the world and present a weighty socio-economic burden on an already strained global health system[1,17]. Cardiac tissue engineering has two distinct goals: (1) to regenerate damaged myocardium after ischemic disease or cardiomyopathy or (2) to create a high fidelity model of the heart for *in vitro* drug screening or disease modeling.

The heart is a complex organ that must work constantly to supply blood to the body. Densely packed laminar structures of cardiomyocytes and supportive tissues are arranged in helical patterns throughout the heart wall[18,19]. The heart is also electromechanically coupled[20] in a highly coordinated fashion to efficiently eject blood to the body[21]. Several major hurdles remain to be addressed, however, before nature's intricate design can reliably be recapitulated *in vitro*. First, although robust cardiomyocyte differentiation methods continue to be developed[22], hPSC-CMs still exhibit rather immature phenotypes. Their electromechanical properties and morphology most closely match fetal levels[23]. Second, when kept in traditional culture conditions, both stem cell-derived and primary cardiomyocytes fail to assemble into native, tissue-like structures. Rather, cells become randomly oriented and do not exhibit the banded rod-shaped appearance of adult myocardium[24].

The extracellular matrix (ECM) environment with which cells interact plays a significant role in numerous cellular processes[11,13,25]. The ECM consists of complex, well-defined molecular and topographical cues that significantly influence the structure and function of cells[6,26]. Within the heart, cellular alignment closely follows the underlying nanometer scale ECM fibers[2]. The impact of these nanotopographical cues on cell and tissue function, however, is far from completely understood. Preliminary studies of nanometer scale cell-biomaterial interaction indicate the potential importance and impact of sub-micron topographical cues for cell signaling[27], adhesion[28-30], growth[31], and differentiation[32,33]. However, due to the difficulty in developing reproducible and scalable nanofabricated substrates, such studies could not reproduce the multi-scale cellular

effects of the complex *in vivo* ECM environment. In this protocol, a straightforward and cost-effective nanofabrication technique to produce cell culture scaffolds mimicking native cardiac ECM fiber alignment is described, allowing for a wide range of novel investigations of cardiomyocyte-biomaterial interactions. Understanding how cardiomyocytes interact with the nanoscale ECM environment could allow for the ability to control cellular behavior to more closely mimic native tissue function. Furthermore, cell monolayers are a simplified experimental system compared to 3D structures but still exhibit complex multi-cellular behavior for insightful investigations and functional screening[2,34-36]. Finally, such scaffolds could be used to improve cellular graft function when implanted into the heart for regenerative purposes[37].

Protocol

All procedures are conducted at room temperature (~23 °C) unless otherwise noted.

1. Fabrication of Silicon Master

1. Clean silicon wafer with 100% ethanol or xylene and dry under O_2/N_2 gas.
2. Place silicon wafer in spin-coater at rotation speeds of 2,000-4,000 rpm to produce a 0.3-0.5 µm thick film.
3. Pattern the photoresist film with the correct dimensions by using a photolithography system
4. Fully immerse the patterned photoresist-coated silicon wafers in an appropriate volume of photoresist developing solution.
5. Rinse the developed photoresist-coated silicon wafers with deionized water.
6. To form arrays of sub-micron scale ridges with near vertical side walls, deep reactive ion etch the exposed silicon using an etching system.
7. Remove the remaining photoresist by placing the silicon wafer in a plasma asher system.
8. Cut the silicon wafers with a diamond-tipped cutter into the appropriately sized silicon masters for subsequent replica molding.

2. Fabrication of PUA Mold from Silicon Master

NOTE: Volume to be added to silicon master for nanofabrication will vary depending on the area of the nanopatterned master to be replicated as well as the viscosity of the polyurethane acrylate (PUA) solution.

1. Clean silicon master surface with 100% ethanol or xylene and dry under O_2/N_2 gas.
2. Place silicon master pattern side up in a Petri dish.
3. For a silicon master with a 2 cm x 2 cm surface pattern, pipette 40 µl of PUA to the pattern surface.
4. Place a sheet of 4 cm x 4 cm transparent polyester (PET) film over the dispensed PUA.
5. Press down on the PET sheet and spread the PUA underneath the sheet across the pattern face using a roller or flat edged surface (such as a card) so that the entire pattern is covered by the PUA prepolymer.
6. Place silicon master, prepolymer, and PET approximately 10 cm below a 20 Watt (115 V) UV light (λ = 365 nm) for 50 sec. To be effective, the UV light wavelength can be anywhere between 310-400 nm. The intensity of the light is 10-15 mW/cm^2 at the surface of the substrate.
7. After curing, remove PET film slowly with forceps. PUA should attach to the PET film with a negative of the silicon master nanopattern.
8. Cure PUA/PET nanopatterns under UV for at least 12 hr prior to use. Overexposure is not an issue.
9. To clean silicon masters, place another film of PET on top of the master without the addition of PUA and expose to UV light (λ = 365 nm) for 50 sec and remove PET film. This will remove any unreacted monomers.
10. Rinse silicon master with 100% ethanol or xylene and dry under O_2/N_2 gas.

3a. Nanopatterning Polyurethane Polymer

1. Prepare 25 mm diameter circular glass slides by placing in an ozone treatment chamber for 10 min.
2. Place ozone-treated glass slides onto small PDMS block for easy handling.
3. Apply thin layer of surface adhesion promoter with paintbrush to glass slides. Air dry glass slides for 30 min.
4. Place the glass slide on a piece of printer paper.
5. Drop dispense 10 µl of polyurethane (PU) pre-polymer (NOA 76) to center of glass slide. Make sure no bubbles are present after addition.
6. Place PUA mold, pattern face down, onto the glass slide. Disperse the PU uniformly across the surface of the glass slide by rolling a rubber cylinder roller along the PUA mold. Printer paper will absorb polymer overflow.
7. Watt UV lamp. Polymerization time of the PU is dependent on power of UV source.
8. Remove the sample from UV light source and carefully peel the PUA mold from PU coated glass slide. Polymerization of the PU is considered complete when the PUA mold peels cleanly away from the sample and the PU glass slide has an iridescent appearance.
9. Place finished samples in desiccator for storage for as long as a month.

3b. Nanopatterning Poly(Lactide-Co-Glycolide) Hydrogel

1. Create a flat PDMS mold by vigorously mixing silicone elastomer base and silicone elastomer curing agent in a 10:1 ratio.
2. Pour mixed PDMS precursor solution into a Petri dish so that the PDMS precursor reaches 5 mm up the edge of the dish (*i.e.* so that the PDMS is 5 mm thick).
3. Place Petri dish and PDMS precursor in a desiccator for 1 hr to degas.
4. Move Petri dish and PDMS precursor into a 65 °C oven for at least 2 hr to cure.
5. After PDMS is cured, use a razor to cut the flat PDMS into 3 cm x 3 cm square sections. These will be the flat PDMS molds used later in the patterning process.
6. Clean 25 mm diameter circular glass slides by placing in isopropyl alcohol for 30 min in a water sonicator.
7. Dry cleaned glass slides under O_2/N_2 gas.
8. Drop dispense 100 µl of PLGA solution (15% w/v PLGA in chloroform) onto glass slide.

9. Place a flat PDMS mold on top of the dispensed PLGA to absorb the solvent and obtain a flat PLGA surface. Apply a light pressure (~10 kPa) by placing a 200 g weight on top of the PDMS for 5 min.
10. Slowly peel away flat PDMS mold and place the cover glass on a preheated plate (120 °C) for 5 min to remove residual solvent and increase adhesion between the PLGA and the cover glass.
11. Place the NP PUA mold on top of the flat PLGA and apply constant pressure (~100 kPa) and heat (120 °C) by placing a 1 kg weight on top of the PUA mold while on the heating plate for 15 min.
12. Remove the weight from PLGA cover slide and allow the substrata to cool to room temperature. Do not remove PUA mold until the substrata have been cooled.
13. Once the substrata have sufficiently cooled, carefully peel away the NP PUA mold, revealing the NP PLGA substratum. The NP PLGA substratum should have an iridescent appearance.
14. Place finished samples in desiccator for storage for as long as a month.

4. Cell Seeding and Culture

NOTE: This protocol describes the culture of neonatal rat ventricular myocytes (NRVMs) and H7 human embryonic stem cell-derived cardiomyocytes (hESC-CMs) but other cell sources may be used.

1. Attach the ANFS coverslips (PU or PLGA) to a 35 mm tissue culture polystyrene dish. Pipette 20 µl of Norland Optical Adhesive (NOA83H) to the bottom of the dish and gently place the ANFS coverslip on top of the NOA. Allow glue to spread out and cover entire coverslip bottom. Cure NOA by exposing dish to UV for 10 min.
2. Sterilize ANFS by rinsing with 2 ml of 70% aqueous ethanol solution for 5 min, twice. Remove ethanol by aspiration. Allow ANFS to completely air dry for ~1 hr under the UV sterilization lamp (λ = 200-290 nm) in the biological safety cabinet.
3. Cellular adhesion is enhanced by coating the ANFS in fibronectin overnight. Dilute fibronectin in DI water to 5 µg/ml. Pipette 2 ml of fibronectin solution into dish. Place in incubator at 37 °C and 5% CO_2 overnight (at least 6 hr).
4. Obtain NRVMs, hESC-CMs, or other cardiac cells of interest according to previous protocols[22].
5. Centrifuge cell sample at 1,000 rpm for 3 min to pellet the cells.
6. Carefully remove the supernatant by aspiration. Make sure not to disturb the pellet.
7. Resuspend cells in appropriate culture media to a concentration of 4.6 x 10^6 cells/ml.
8. Carefully pipette 200 µl of cell suspension onto sterilized ANFS. Make sure cell suspension remains on the coverslip.
9. Place cells in incubator at 37 °C and 5% CO_2 for 4 hr to allow cells to attach to ANFS.
10. Add 2 ml of warm culture media to dish and replace cells in incubator under the same conditions.
11. After 24 hr, remove media and wash with 2 ml of DPBS twice to remove excess cells.
12. Add 2 ml of warm culture media to dish and replace cells in incubator under the same conditions. Culture the cells to confluence. Replace media every other day.

Representative Results

Figure 1 is a schematic overview of the production process for the two fabrication methods. Due to the diffraction of light caused by the nanoscale topography, nanopatterning should result in an iridescent surface to the ANFS. **Figure 2** depicts this iridescent surface on a well-patterned 25 mm NP-PU coverslip **(Figure 2A)** with 800 nm ridge and groove width **(Figure 2B)**. The iridescent appearance of the ANFS will vary slightly depending on the ridge and groove widths.

After seeding the cells onto the ANFS, cells should begin to align in the direction parallel with the ridges of the substrate. Alignment should become apparent within the first 24 hr after seeding and should remain for the entirety of the culturing period. **Figure 3** shows representative bright field images of NRVMs after 7 days of culture and hESC-CMs after 48 hr of culture. NRVMs and hESC-CMs on the NP surfaces show obvious structural anisotropy and alignment (**Figures 3B** and **3D**) whereas cardiomyocytes on unpatterned surfaces are randomly oriented (**Figures 3A** and **3C**). Immunohistochemical analysis highlights the impact of the nanotopography on cell cytoskeletal alignment. Actin microfilaments (F-actin) and α-sarcomeric actinin in cells cultured on the ANFS become aligned along the nano-ridges but remain randomly distributed in cells on unpatterned substrata (**Figure 4**). Cardiac cells exhibit spontaneous beating after 24-48 hr of culture.

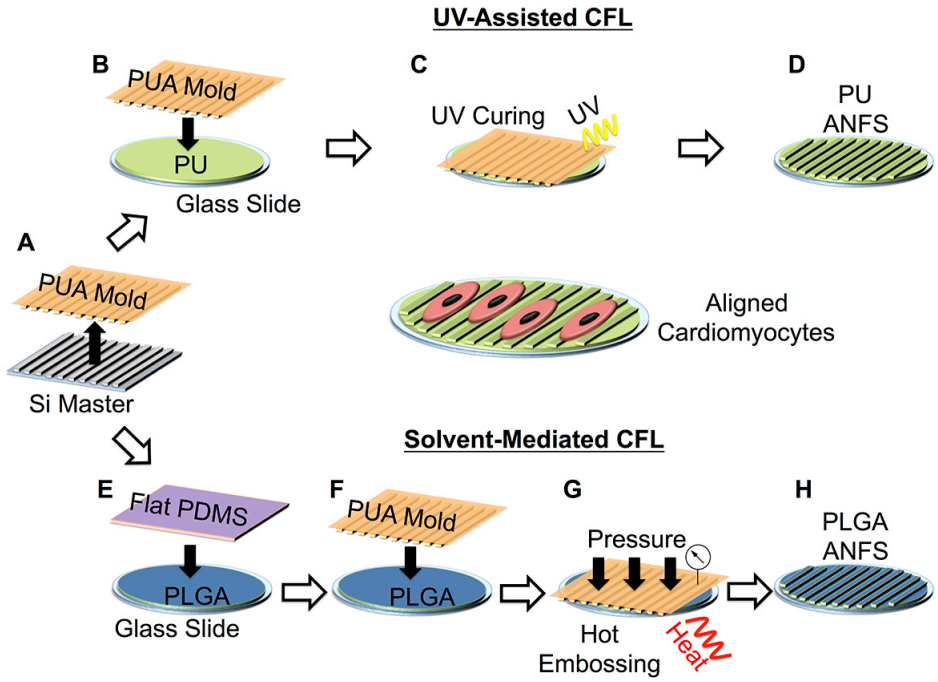

Figure 1. **Nanofabrication schematic** – diagram of the two nanofabrication processes. (**B**-**D**) Depict UV-assisted capillary force lithography (CFL), while (**E**-**H**) depict solvent-mediated CFL. (**A**) PUA negative is made from a silicon master. (**B**) PU pre-polymer is drop-dispensed onto a glass slide and the PUA mold is placed on top. (**C**) A PUA mold and PU coverslip are then exposed to UV light to cure the PU. (**E**) PLGA polymer solution is drop-dispensed onto a glass slide and a flat PDMS mold is used to create a flat PLGA surface. (**F**) A PUA mold is placed on top of the flat PLGA and (**G**) constant heat and pressure are applied to hot-emboss the NP into the PLGA. (**D**,**H**) After peeling away the PUA mold, a NP-PU or PLGA substrate is left behind. Cardiac cells can then be seeded onto this NP surface to create aligned arrays of cells.

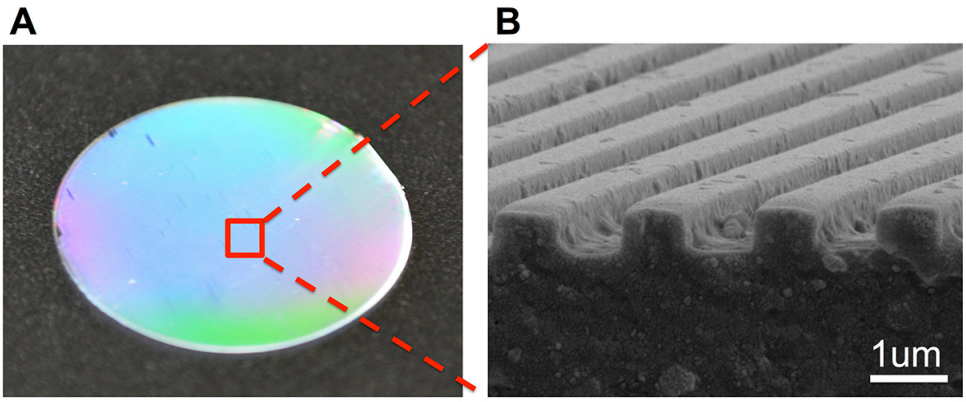

Figure 2. NP-PU substrate. (**A**) Photograph of large area NP surface. The iridescent appearance of the coverslip is caused by the nanotopography. (**B**) SEM image of the cross-section of the underlying nanotopography.

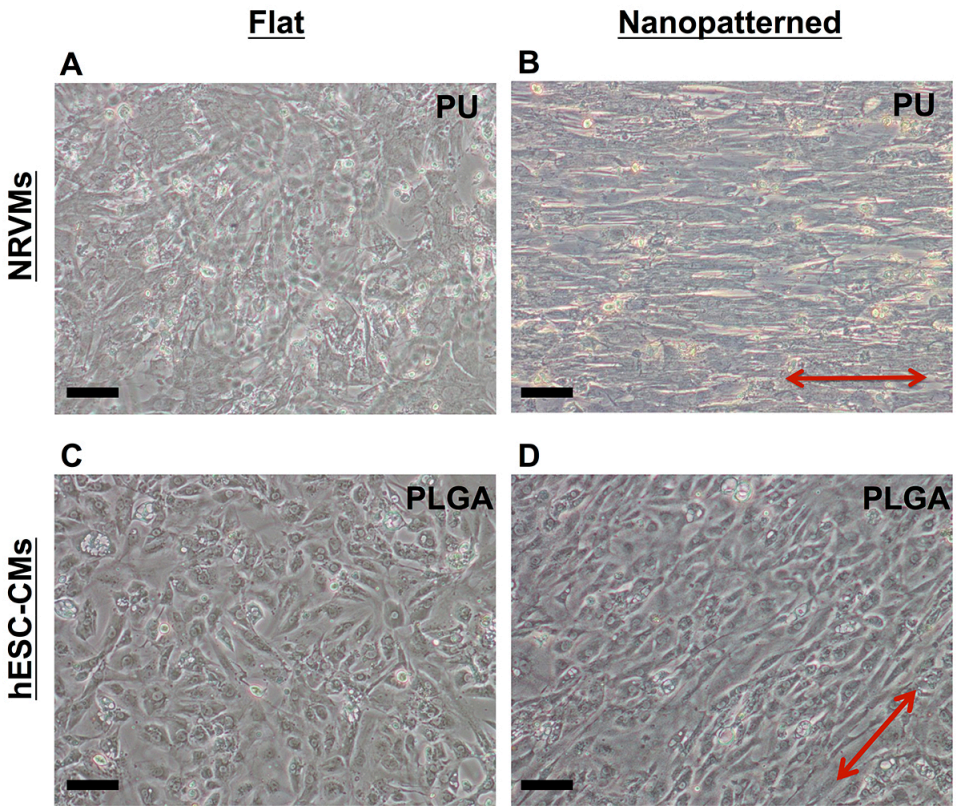

Figure 3. Aligned cardiomyocytes. 10X bright field images of NRVMs after 7 days of culture (**A**,**B**) and hESC-CMs after 2 days of culture (**C**,**D**). NRVMs cultured on NP-PU substrates (**B**) exhibit obvious structural alignment while NRVMs on flat PU substrates (**A**) are randomly aligned. Arrows in (**B**) and (**D**) indicates direction of NP anisotropy. Scale bar = 100 μm.

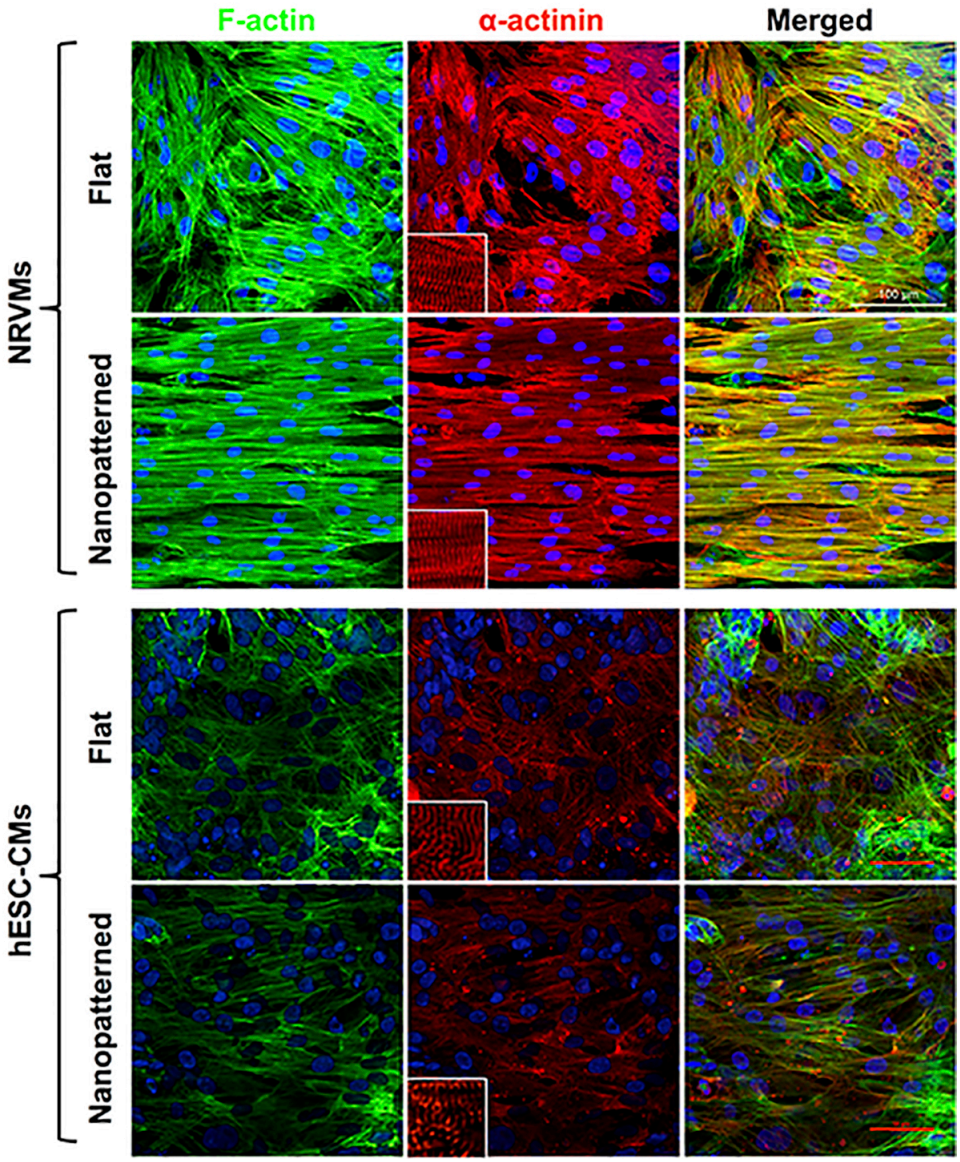

Figure 4. Immunofluorescent confocal imaging. Cell cytoskeletal alignment and striations; α-sarcomeric actinin (red), cell nuclei (blue), and F-actin (green). Cells cultured on NP substrates have aligned α-sarcomeric actinin and F-actin fibers while cells cultured on flat substrata have randomly oriented fibers. Inset on red α-sarcomeric actinin channel clearly show striated sarcomeres.

Discussion

Functionally mature cardiac tissues are lacking for both *in vivo* and *in vitro* applications of cardiac tissue engineering. The CFL nanofabrication methods described here are robust techniques for achieving cellular alignment and influencing macroscopic tissue function due to the scalability of the system. Large areas can easily be patterned and used for cell culture. Macroscopic cellular alignment is essential in cardiac tissue engineering in order to create biomimetic, functional tissue as it influences both mechanical and electrical properties of the myocardium[38].

The methods here can be applied to a wide range of applications within cardiac tissue engineering. It has previously been demonstrated that nanoscale cues help to create a cardiac stem cell niche and promote regeneration[14]. Thus by using biodegradable polymers such as PLGA, nanopatterned “patches” could be made to promote healing after cardiac insult. Alternatively, by using hydrogels with similar elastic moduli as the native heart, cardiomyocyte-ECM interactions can be studied in a simplified, reproducible system.

Various other methods, such as microcontact printing, electrospinning, and microtopography, have previously been employed to control the structure of engineered cardiac tissue on the microscale[39-41]. While these techniques have proven successful in gaining cellular alignment, it is likely that the structure and function of *in vivo* cardiac tissue is governed by the much smaller, nanotopographical cues of the ECM and thus our NP substrate should prove advantageous. Additionally, these microscale patterning techniques have inherent disadvantages compared to our anisotropically nanofabricated substrates. For instance, our nanopatterning method is more cost-effective and controllable than electrospinning. Microcontact printing, on the other hand, relies on cells adhering to specific lanes to gain structural anisotropy. In order to create a functional monolayer, cells must then either proliferate or migrate out of these lanes to form tight junctions. This is much more difficult for cells with little

to no proliferative capacity such as neonatal cardiomyocytes. Microtopography is also limited by the inability to create tightly coupled cell monolayers. Due to the scale of the cells compared to the microtopography, cells reside on top of or inside the microridges. This limits the amount of cell-cell interaction and decreases cell-cell coupling. With our method, cells become aligned via the bioinspired nano-scale topography and can freely interact with neighboring cells as the feature sizes are at least an order of magnitude smaller than the cells themselves. This allows for more biomimetic, tightly coupled cells monolayers to be created.

For optimum results with the NP-PU we strongly suggest following the glass coverslip pre-treatment steps. These steps will increase polymer adhesion to the glass surface. This will not only aid in the clean removal of the PUA master during fabrication but also prevent the patterned polymer from detaching from the glass during the experiments. To test the effectiveness of the pre-treatment steps, place one NP-PU substrate in water and observe if the polymer remains adhered to the glass.

PLGA is a co-polymer of glycolic acid and lactic acid that was developed for both device and drug delivery *in vivo*. Thus, this substratum provides a good, biocompatible ECM for the cells. The rigidity of PLGA can be modulated by either tuning the ratio of glycolic acid to lactic acid or by altering the contraction of PLGA in chloroform.

Various parameters can easily be varied and adjusted in this system for optimization or investigational purposes. Various PU-based polymers are available with a wide range of mechanical properties. Thus by using different PU pre-polymers, various substrate rigidities can be fabricated by the same protocol. The user can also alter the substrate topography. The nanotopography of the substrate is dependent on the design of the silicon master. Therefore, a variety of ridge and groove widths, as well as various geometries, can be patterned by altering the silicon master design to meet specifications.

The size of the nano-ridges will also influence the cell and tissue behavior. Smaller groove widths allow for less cell penetration into the grooves and limit the cell's interaction with the engineering nanotopography[2]. This in turn will alter the extent of anisotropic behavior of the cardiac cell monolayer. Additionally, the presented protocol is limited to two-dimensional (2D) cell culture and alignment. Obviously, to be truly biomimetic, engineering cardiac tissue would need to be 3D. Future work is needed for designing dense and aligned functional3D cardiac tissue. The protocol presented here, however, offers superior control of cardiac cell morphology and allows for the control of macroscopic cardiac tissue function based on nanoscale cues.

Disclosures

Authors have nothing to disclose.

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