

MECHANISTICALLY MODULATED CARDIOMYOCYTE ALIGNMENT

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ABSTRACT

Substrates with micron-size ridges and grooves correlate with increased orientation of cultured cardiomyocytes. Primary cells from neonatal rats were used in this work. The substrates were prepared with soft-lithographic technique. SU-8 negative photoresist was photolithographically defined to form various patterns with micron-sized precision. Poly(dimethylsiloxane) (PDMS) was molded and cured from the SU-8 masters. Five versions of the substrates were fabricated, each with 5 μm -wide ridges separated by 5, 10, 15, 20, or 30 μm grooves. Cell alignment was quantified with two parameters: percent oriented cells and ratio of the major/minor axis of the fitted ellipses over the cells.

KEYWORDS: Cardiomyocyte, PDMS, Pattern, Alignment

INTRODUCTION

According to the World Health Organization, cardiovascular diseases (CVD) are the number one cause of death globally, claiming 17.7 million lives in 2015, representing 31% of all global deaths [1]. The studies of cardiomyocytes in vitro, particularly the biomechanics of the cells under various stimuli, have been generating scientific insights beyond the traditional electrophysiological approaches [2–5]. The motivation of a large body of similar research ultimately is to develop a platform for drug development for CVD and screening for cardiotoxicity with cultured cardiomyocytes. Since live human cardiomyocytes are difficult to obtain, most research on this subject are based on induced pluripotent stem cells from human or animal models. Previous studies indicated that topographic features on the culturing substrate can affect cellular response from initial attachment, then differentiation and finally towards the construction of new tissues [6]. It has been demonstrated that micro-fabricated substrates with micron-sized grooves can significantly enhance cell alignment [7], promoting their contractility [8], and impulse propagation along the long axis of the cell bodies [9]. Knowledge on in vitro behaviors and responses to controlled stimuli of cultured cardiomyocytes would enhance the designs of tissue-based platforms for diagnostic and therapeutic applications for CVD.

EXPERIMENTAL

This paper demonstrates how support structures of micron size ridges and grooves correlate with increased orientation of cultured cardiomyocytes. In this work, neonatal rat ventricular myocyte (NRVM) primary cells harvested from two-day old Sprague Dawley rats were used. In contrast, our previous work [10] was based on purchased HL-1 cardiac muscle cells derived from AT-1 mouse atrial cardiomyocyte tumor lineage [11] (ScienCell Research Laboratories, Carlsbad, CA). The results were compared with a plan for improving the micro-pattern alignment technology. The ability for controlling the micro-environment of cardiac cells at the micro-scale can provide further mechanical cues on the functional assembly of this particular tissue model.

A set of patterned substrates was designed each with 5 μm -wide ridges separated by 5, 10, 15, 20, or 30 μm grooves. These patterns were created on a film mask with 20,000 dpi resolution (CAD/Art Services, Bandon, OR), with which a one-mask photolithographic process was performed to create masters with SU-8 2000 negative photoresist (MicroChem Corp., Westborough, MA). Multiple masters were created with three different thicknesses of SU-8: 5, 7.5, and 10 μm . Three sets of PDMS devices were then molded from the SU-8 master. Process details can be found in [12]. Figure 1 shows the 5, 15, and 30 μm versions. Prior to seeding, the PDMS devices were all autoclaved, followed by coating with gelatin fibronectin to promote cell adhesion. Figure 2 shows the illustrative cross section of the experiment.

Figure 3 shows the photomicrographs of the NRVM cells studied in this work. These cells were seeded in gelatin fibronectin-coated PDMS devices and cultured in specific complete media. For indirect immunofluorescence, the cardiomyocyte cells were fixed and permeabilized with 4% paraformaldehyde and 0.1% Triton X-100 in a PBS (pH 7.2) for 15 minutes at room temperature. The primary antibodies used for staining included DAPI to observe the nuclei and Phalloidin to examine the actin within the cardiomyocytes.

RESULTS AND DISCUSSION

The fluorescent imaging result is shown in Figure 4(A). An orientation analysis was conducted using the image processing routines in MATLAB® and ImageJ. The patterned grooves from the brightfield images were aligned vertically, which were then cropped to 850x700 pixels in size. All images were adjusted for brightness/contrast and corrected for noise. To capture the cells' outlines, the images were binarized [Figure 4(B)] and each of the outlines fitted to an elliptical shape. Ellipses with an area less than 10 pixels were discarded from the dataset [Figures 4(C) and (D)].

Two parameters were investigated to quantify the orientation: (1) percent oriented cells and (2) ratio of the major/minor axis of the fitted ellipses. A cell with a fitted ellipse that aligned perfectly with the vertical line (with the direction of the ridges) would be assigned 90° . Any cell oriented within the range of 75° to 105° was considered aligned. The percentage of all cells considered aligned constituted the first parameter. The ratio of the major/minor axis was to be used to further quantify the alignment since a more elongated cell body would be more prominently aligned. All five versions of the PDMS devices increased cell orientation when , and on an average of 56%, with the $20\ \mu\text{m}$ version exhibiting the most significant alignment results.

When comparing the results between NRVM cells in this work with our previous work with HL-1 cells [10], no statistically significant difference was observed in alignment properties. However, it was observed that contraction appeared to be more frequent and the magnitude of contraction appeared stronger with NRVM cells than with HL-1 cells. The key findings that both NRVM cells and HL-1 cells responded similarly to mechanical cues from the substrates should be explored further for cell-based biomechanics experiments.

CONCLUSION

A set of substrates with different ridge-and-groove patterns fabricated with PDMS proved to be useful in studying the mechanistic responses of cardiomyocytes to environmental mechanical cues. It was found that under controlled conditions, both NRVM cells and HL-1 cells responded similarly to these mechanical influence without statistically significant difference. It was further observed that NRVM cells appeared to contract more often and more strongly than HL-1 cells. Further studies are warranted from these findings to compare these two cell types with more intricate mechanical stimuli.

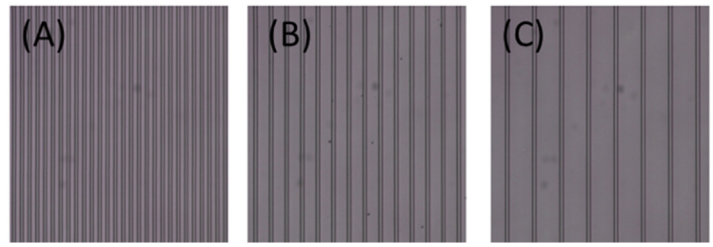


Figure 1: Photomicrographs at 40x magnification of PDMS devices with $5\ \mu\text{m}$ wide ridges spaced at (A) $5\ \mu\text{m}$, (B) $15\ \mu\text{m}$, and (C) $30\ \mu\text{m}$.

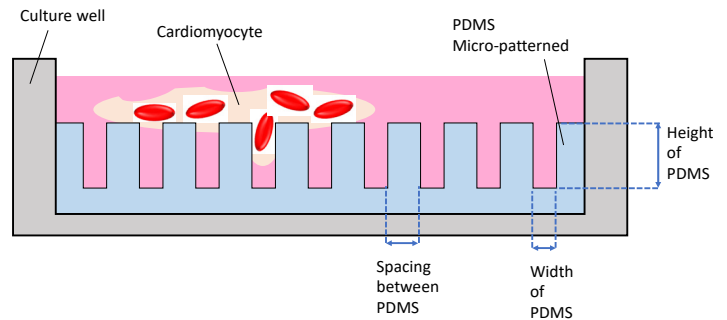


Figure 2: Illustration of the cross sectional view of the experimental setup.

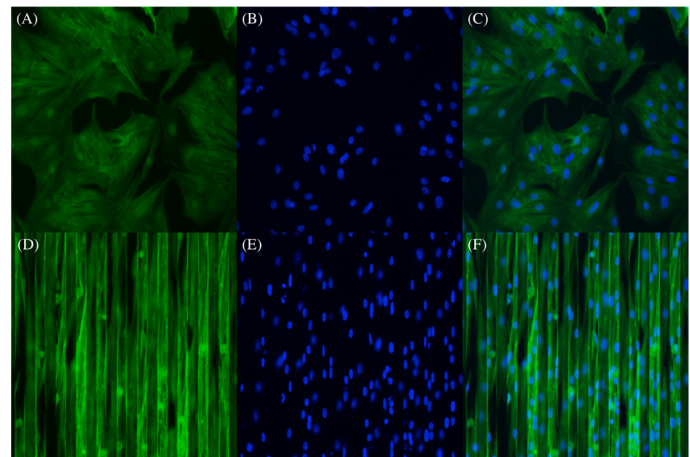


Figure 3: Photomicrographs at 20x magnification of NRVM after 3 days in culture. Control groups (A–C): cells seeded on blank PDMS (A) Stained with phalloidin to observe actin of the NRVM. (B) Stained with DAPI to observe the NRVM nuclei. (C) Merged image of (A) and (B). Experimental groups (D–F): cells seeded on a $20\ \mu\text{m}$ ridge at $20\ \mu\text{m}$ separation micro-patterned PDMS (D) Stained with phalloidin to observe the actine if the NRVM. (E) Stained with DAPI to observe the nuclei of NRVM. (F) Merged image of (D) and (E).

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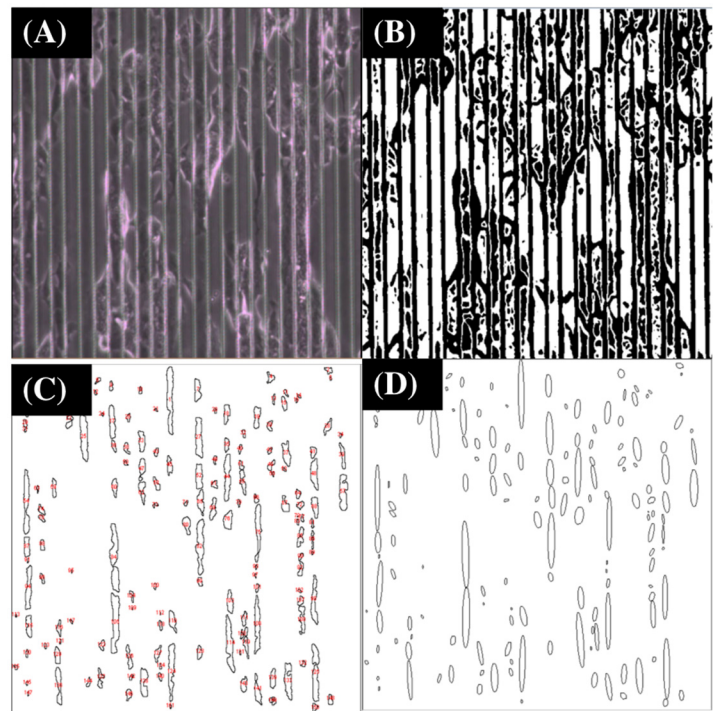


Figure 4: (A) Original photomicrograph two days after seeding onto patterned PDMS substrate. (B) Completed binarization with ImageJ, forming the outlines of the cellular membranes. (C) Cellular outlines from automatic particle analysis algorithm from ImageJ. (D) Elliptical fitting to the outlines generated, which were then used to assign orientation angle and elongation ratio.