
Microfabricated grooves recapitulate neonatal myocyte connexin43 and N-cadherin expression and localization

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Received 31 July 2002; revised 3 January 2003; accepted 2 February 2003

Abstract: Our objective is to alter the surface topography on which cardiac myocytes are grown in culture so that they more closely resemble their *in vivo* counterparts. Microtextured silicone substrata were made using photolithography and microfabrication techniques and then coated with laminin. Primary cardiac myocytes from newborn rats were plated on microgrooved and nontextured substrata. Myocytes were highly oriented on 5 μm grooves ($69.8 \pm 2.0\%$) and significantly different, $p < 0.0001$, compared with randomly oriented cells grown on nontextured surfaces ($2.9 \pm 0.95\%$; $n = 19$). Cells on shallower, 2 μm , grooves were slightly less well oriented ($46.9 \pm 4.3\%$, $n = 5$, $p < 0.001$). The lateral spacings of the grooves were altered to examine changes in cell-to-cell contact by confocal immunocytochemistry and quantitative protein analysis. Connexin43 and N-

cadherin were distributed around the perimeter of the myocytes plated on $10 \times 5 \times 5$ microgrooved surfaces, similar to the localization found in the neonate. Connexin43 expression in cultures on 5 μm deep grooved substrata was equal to the neonatal heart, whereas it differed in nontextured surfaces. We conclude that it is necessary to combine groove depth (5 μm) and lateral ridge dimensions between grooves (5 μm) in order to recapitulate connexin43 and N-cadherin expression levels and subcellular localization to that of the neonate. © 2003 Wiley Periodicals, Inc. *J Biomed Mater Res* 67A: 148–157, 2003

Key words: microtopography; tissue engineering; connexin43; N-cadherin; cardiac; myocyte

INTRODUCTION

The objective of this article was to use photolithographic techniques to microfabricate three-dimensional microgrooved substrata that would permit cardiac myocytes grown in culture to resemble their *in vivo* counterparts more closely. The heart is a vital organ and manipulations of function *in vivo* could result in the death of the animal; therefore, many of the experiments designed to understand fundamental principles in cardiac physiology are performed *in vitro*, even though present cultures are far from ideal. The heart is a dynamic organ in that it remodels to

serve the changing demands of the body. These changes occur at many levels, including at the level of the myocyte. The regulation of cell shape remodeling, although not well-understood,¹ holds the key to discerning some of the cell's fundamental operations.

Cellular orientation has been studied in a number of cell types using superficial imprinting, texture and collagen streaking to align cells.^{2,3} Documented primarily in fibroblasts, alignment reflects the cell's ability to respond to changes in surface pattern in its environment. The finding that fibroblasts change in shape and orientation when plated on textured surfaces led to the principle of contact guidance.^{4,5} Microfabrication has proven to be a powerful tool in that it enables the investigator to manipulate the surface microarchitecture from the micron down to the nanometer scale. Textured environments occur naturally *in vivo* to orient cells in tissues but such guidance can be made available to the cells *in vitro* through the use of microfabricated topographical structures. Orientation of various cell types has been found to increase as groove depth increased within the range of 1 μm to 25 μm (for review, see Ref. 3). In agreement with other findings,^{2,3} we found that an increase in groove depth resulted in an increase in cell alignment. Parallel mi-

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Contract grant sponsor: National Institutes of Health; contract grant numbers: HL64956, HL 62426 (to B.R.)

Contract grant sponsor: National Science Foundation NRSA; contract grant number: Career Grant BES9983840 (to T.D.)

Contract grant sponsor: National Institutes of Health; contract grant number: T32 HL 07692 (to D.M.)

crogrooved substrata of 5 μm depth seem to best mimic the arrangement present in the neonatal heart.

Alignment is necessary for effective force transmission and also to allow the normal cell-cell contact, which is critical in maintaining a viable culture environment.⁶ Myocytes are connected end-to-end by intercalated disks providing both electrical and mechanical communication.⁷ The intercalated disks strengthen the adhesion between myocytes allowing for transmission of force between contractile units. In the rat heart, the myocytes are linked with adjacent neighbors electrically by connexin43, the major gap junction protein in the heart, and mechanically by N-cadherin and other fascia adherens proteins. Morphological studies have noted changes in the distribution of connexin43 and N-cadherin proteins as the heart develops. In the neonate, gap junctions and fascia adherens are distributed in a relatively uniform manner along the perimeter of the myocytes,^{8,9} whereas, in the adult, they are located primarily at the ends of the cells and the area lateral to the intercalated disks.¹⁰ The mechanism of the changes in connexin43 and N-cadherin localization in the developing heart are not well understood. As the myocardium matures to that of the adult phenotype, the longitudinal conduction velocity increases whereas the transverse velocity remains unchanged,¹¹ suggesting that the neonate is less electrically anisotropic. Having established the range of groove depth, we explored lateral spacings (in the range of 5–10 μm) in order to allow myocytes to make more life-like cell-to-cell connections.

Our findings with textured surfaces show alteration in the morphology of the myocytes when cells are aligned along the parallel microgrooves. The microgroove dimensions were systematically varied in order to optimize both myocyte orientation and cell-cell contact. In order to optimize the topography, we calculated the percent orientation of myocytes plated on microgrooved surfaces with depths of 2–5 μm and groove and ridge widths of 5–10 μm . Myocytes were examined for electrical and mechanical junctional protein expression and localization. The functional consequences of these reconnected myocytes were assessed by synchronicity of beating. Gene expression, anatomical features, and physiological function determined the optimal topography.

MATERIALS AND METHODS

Production of templates and polydimethylsiloxane surfaces

The fabrication process for creating microgrooves has been previously reported.¹² The spacings of the grooves were adjusted as desired according to the mask pattern. Briefly, approximately 1 mL of positive photoresist (Shipley

1818) was spun on a clean 2-inch diameter silicon wafer at 500 rpm for 180 s. After a 5-min soft bake at 100°C, the wafer was patterned using a mask aligner for 13 s at 20 mW. This process resulted in features with oriented lines of varied depth. The depth of the grooves could be altered by the spin speed at which the photoresist was deposited. The wafers were placed in developer (Shipley 351), with continuous motion for 54 sec and rinsed in deionized water. The patterned wafers were then placed in the parylene vapor deposition chamber (Labcoater 1 PDS 2010). The purpose of parylene was twofold: 1) so a reusable template would be formed and 2) to allow for easy separation of the silicone polymer gel from the silicon wafer. All surfaces were coated with a release solution, MICRO-90 soap solution which contains water; Glycine, *N,N'*-1,2-ethanediybis (*N*-(carboxymethyl)-, tetrasodium salt; Benzenesulfonic acid, dimethyl-ammonium salt; Benzenesulfonic acid, dodecyl- cpd . With 2,2',2'-nitrilotris(ethanol); Poly(oxy-1,2-ethanediy), α (undecyl)- ω -hydrox) to allow easy parylene removal from both the wafer surfaces and the surface of the deposition chamber. Parylene dimer (Specialty Coating Systems) was placed in the vaporizer chamber. The parylene was heated to vaporization and monomerization temperature (690°C), then cooled as it entered the deposition chamber, with the wafers, and polymerized coating every surface. The run was complete when the furnace temperature started to come back down from 690°C. The parylene template was then cut away from the wafer and used as a mold for polymer membranes. A 25 μm thick parylene layer was deposited on the patterned photoresist and then peeled off, resulting in a parylene microtextured mold, which could easily be peeled away from any polymer and was reusable several times. Silicone [polydimethylsiloxane (PDMS)] was prepared by mixing elastomer and catalyst (A103 Factor II, Inc.) in a 10:1 ratio. The wet mixture was poured over the desired parylene template and allowed to cure 2–3 days at room temperature or overnight at 37°C. The silicone membranes were then peeled and treated with 12M HCl for 1–2 h at room temperature. After the acid treatment, the membranes were rinsed 2–3 times with distilled water and left to dry at 37°C overnight. The membranes were soaked in ethanol for at least 1 h followed by exposure to UV light in the hood for 1 h in order to sterilize the surface. The membranes were then rinsed with Moscona's saline (136.8 mM NaCl, 28.6 mM KCl, 11.9 mM NaHCO_3 , 9.4 mM glucose, 0.08 mM NaH_2PO_4 , pH 7.4) and incubated in a solution of laminin (25 $\mu\text{g}/\text{mL}$) in DMEM (Dulbecco's modified Eagle's medium nutrient mixture, Sigma, St. Louis, MO) overnight at 37°C, in a 5% CO_2 incubator.¹³ The laminin-coated membranes were rinsed with warm Moscona's saline before use.

Cell isolation

Primary myocytes were obtained from neonatal rats according to Institutional Animal Care and Use Committee and NIH guidelines for the care and use of laboratory animals (NIH publication 85-23, Rev. 1985). Isolation of primary cardiac myocytes and culturing was previously described.¹⁴ Briefly, hearts were removed from 1–2-day-old neonatal Sprague-Dawley rats and kept in cold Moscona's

saline on ice. Ventricles were trimmed free and minced with dissecting scissors. Cells were dissociated in a 37°C shaker bath with collagenase (type 2, 0.42 mg/mL from Worthington Biochemical Corporation) in Krebs-buffered Ringer (118.4 mM NaCl, 2.4 mM MgSO₄, 4.7 mM KCl, 23.8 mM NaHCO₃, 1.5 mM KH₂PO₄, 11.1 mM glucose) to which was added 20 mg/mL BSA Fraction V, 10 μL/mL penicillin G/streptomycin (Sigma) and Phenol Red. This solution was gassed with 5% CO₂ to achieve pH 7.4. Cells were pelleted by centrifugation at 5000 rpm for 6 min at room temperature. The supernatant was discarded and cells were resuspended in complete media [DMEM F-12 Ham without L-glutamine from Sigma, standard amino acid concentrations plus palmitic (2.56 mg/L) and linoleic fatty acids (0.84 mg/L), penicillin G/streptomycin (10 μL/mL), and gentamicin (60 mg/L)] and then filtered through a metal sieve to filter out large chunks of material. The resulting mixture was preplated for 1 h in 37°C, CO₂ incubator. Cells still in suspension were enriched with myocytes and were plated on laminin-coated silicone substrata (as previously described¹²) at very high density (1000–2000 cells/mm²) and maintained in complete media (as defined above) with 5% fetal bovine serum for 4 days.

Orientation

Rat neonatal cardiac myocytes were plated on textured and nontextured substrata after isolation and allowed to attach overnight. The substrata were rinsed with fresh media to remove debris and maintained for a total of 4 days. Micrographs were taken of myocytes in culture on day 4 using a Nikon TMS phase-contrast microscope. All fields were selected randomly and images taken at the same magnification using a 20× objective. A parallel lined grid with 50-μm spacing was placed first parallel with the grooves (0°) and then perpendicular to them (90°). A count, A, was taken of the number of intersections between the grid lines and the cell edge. Another count, B, was taken by intersections made between the 90° lines and the cell edge. The percent orientation was computed by the following formula:

$$\% \text{ orientation} = \frac{B - A}{B + A} \times 100$$

Immunolabeling and confocal microscopy

Neonatal cardiac myocytes were washed in phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde/PBS for 10 min, as previously described.¹⁴ N-cadherin and connexin43 were detected with monoclonal antibodies (BD Transduction Laboratories, San Diego, CA) diluted at 1:500 and 1:100, respectively. The cells were rinsed in PBS and incubated with a FITC-labeled secondary antibody (1:500). Actin filaments were stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR), which recognizes all isoforms of filamentous actin. Confocal microscopy (Zeiss LSM510) was utilized to obtain optical sections of the myocytes from the bottom of the groove to the

top of the cell, Z-stack. Sections from whole neonatal hearts were obtained from 1–3-day-old rats. The hearts were removed and embedded in Histo Prep (Fischer Scientific, Brightwaters, NY) and cryosectioned at 5–10 μm. Sections were fixed and immunostained for connexin43 and N-cadherin as described above.

Protein analysis and western blot

Myocytes were rinsed with PBS and scraped in cell lysis buffer (1% SDS and protease inhibitor cocktail; Sigma). Protein concentrations were determined with BCA (Pierce, Rockford, IL) protein concentration assay. Equal amounts of protein were loaded in duplicate onto and resolved by a 7.5% SDS-polyacrylamide gel and transferred by electroblotting onto nitrocellulose (hybond-C extra) membranes (Amersham, Piscataway, NJ). The membranes were blocked with 3% nonfat milk (Carnation, Pickering, ON) in PBS and 0.05% polyethylene sorbitan monolaurate (Tween 20) and probed with appropriate antibodies for N-cadherin and connexin43 used above but now diluted at 1:4000 and 1:150, respectively. The membranes were then incubated with a goat anti-mouse with horseradish peroxidase-conjugated secondary antibody diluted at 1:10,000 (BD Transduction Laboratories, San Diego, CA). The proteins were visualized using chemiluminescence (ECL, Amersham, Piscataway, NJ) and exposed to ECL film (Amersham). Membranes were stripped with 1% SDS, 15 mM Tris buffer, pH 6.8, 0.02% β-mercaptoethanol at 55°C for 30 min and stained with Amido Black staining solution (Sigma). Both stained membranes and ECL films were scanned and analyzed for optical density with Gel-Pro Analyzer 3.0 (Media Cybernetics, Des Moines, IA). All data have been expressed as the densitometric ratio of ECL signal to total protein staining.

Synchronous beating

At 72 h, myocyte beating was evaluated with a 40× objective by time lapse microscopy (Spot RT camera, Diagnostic Instruments) for six or more areas on each surface with counts of both total cell number and number of cells beating asynchronously. Percentage of synchronicity was derived by dividing number of synchronous cells by the total number of cells.

RESULTS

Evaluation of microgrooved substrata

A series of well-defined, three-dimensional parallel grooves were created in PDMS using previously described photolithographic techniques.¹² The desired pattern was initially formed in photoresist on a silicon wafer [Fig. 1(A)]. Once the desired topography was achieved, a parylene template [Fig. 1(B)] was made to

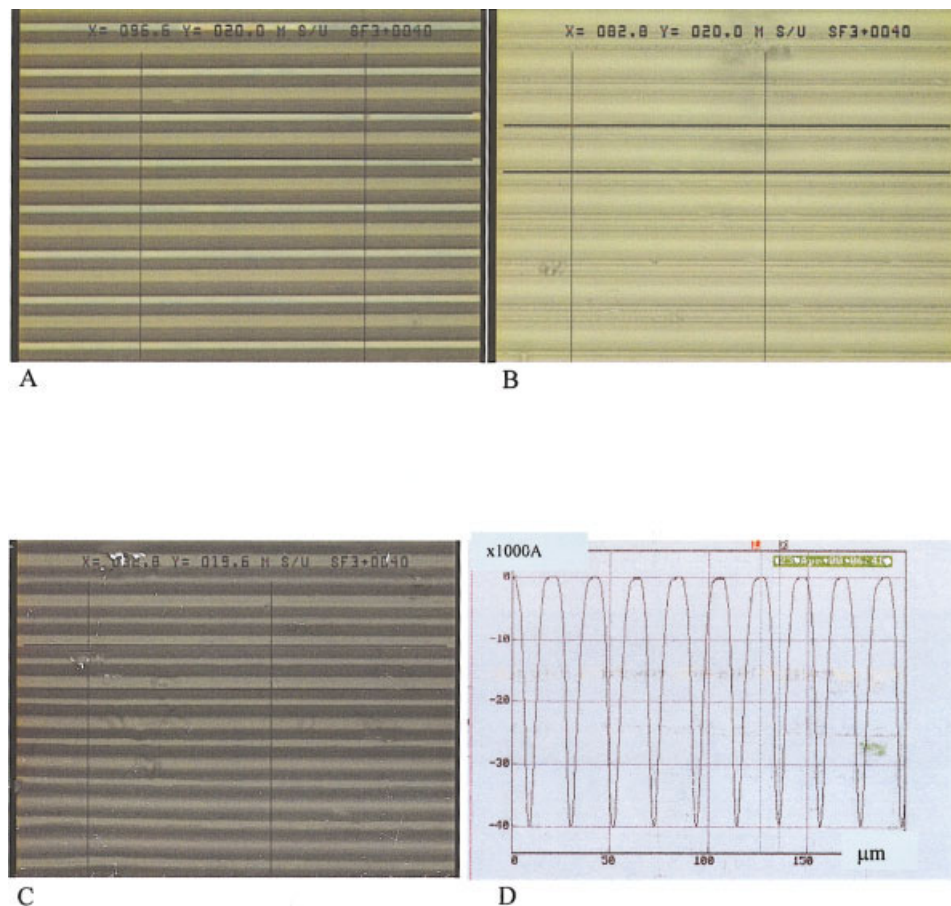


Figure 1. Micrographs showing (A) patterned wafer; (B) parylene template; (C) silicone membrane with grooved microstructure; and (D) profile of template microtexture. Note that the groove depth is $4\ \mu\text{m}$ and the width and spacing of the grooves is $10\ \mu\text{m}$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

serve as a reusable mold for silicone membrane production [Fig. 1(C)]. Profiles were taken after each step in the process to verify the pattern dimensions on the surface, as seen in a representative parylene template profile [Fig. 1(D)]. This process confirmed that the photolithographic process created valid and reliable microtextured grooves. With the assurance that these surfaces could be reproduced faithfully, the microgrooved dimensions were systematically varied in order to optimize the topography for cardiac myocytes from neonatal rats. The initial dimensions set the groove width at $10\ \mu\text{m}$, the ridge separating the grooves at $10\ \mu\text{m}$, and the depth at $5\ \mu\text{m}$ ($10\times 10\times 5$), as depicted schematically in Figure 2(A). In order to facilitate the study of the effects of shallow groove depth, a template was produced with a $10\ \mu\text{m}$ groove, $10\ \mu\text{m}$ ridge, and $2\ \mu\text{m}$ depth [$10\times 10\times 2$, Fig. 2(B)]. The alteration in groove depth was accomplished by adjusting the spin speed at which the photoresist was deposited and was verified by profileometry [Fig. 1(D)]. Groove width was altered to produce a template with a width of $5\ \mu\text{m}$, ridge of $10\ \mu\text{m}$, and a depth of $5\ \mu\text{m}$ ($5\times 10\times 5$, Fig. 2(C)). Finally, the space between the grooves, the ridge, was decreased yielding a tem-

plate with a $10\ \mu\text{m}$ wide groove, $5\ \mu\text{m}$ wide ridge, and $5\ \mu\text{m}$ depth [$10\times 5\times 5$, Fig. 2(D)]. Alterations in the mask pattern are made easily, providing much needed flexibility in adjusting the microgroove dimensions.

Myocyte alignment

The isolation procedure yielded spherical cells that take time to spread and attach on the textured and nontextured surfaces. When examined at 18–24 h after plating, the myocytes had begun spreading on the surface and making contacts with neighboring cells. At 96 h, when the micrographs were taken, the myocytes were beating spontaneously and had made cell-cell contacts within each groove as well as lateral connections between grooves in some cases. In order to establish which topographic factors have the greatest influence in determining cellular orientation, the dimensions of the grooved surfaces were systematically changed. Images of myocytes [Fig. 3(A–E)] were used to analyze alignment on the microgrooved surfaces of different dimensions. As a point of reference,

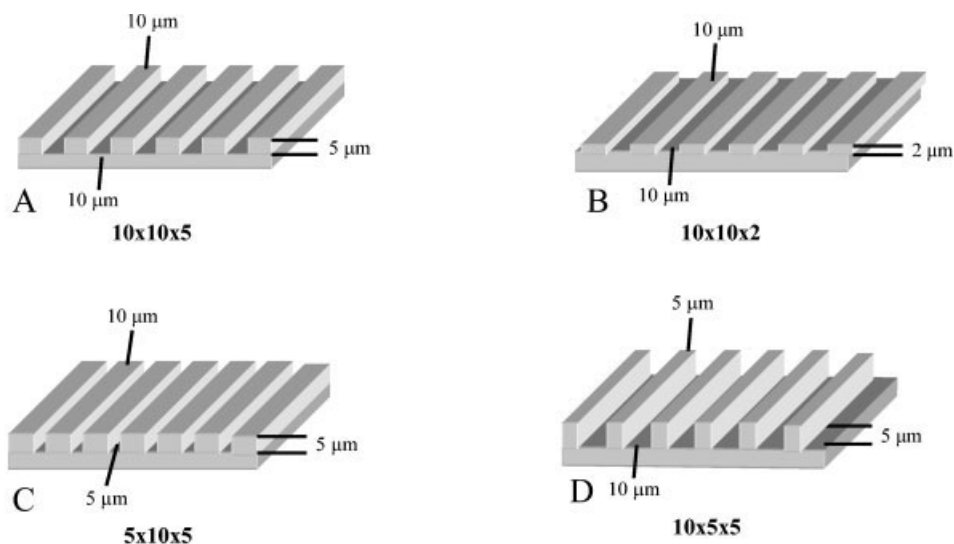


Figure 2. Schematic of microgrooved surfaces defined as groove \times ridge \times depth: (A) $10 \times 10 \times 5$; (B) $10 \times 10 \times 2$; (C) $5 \times 10 \times 5$; and (D) $10 \times 5 \times 5$.

the percent of orientation on traditional flat (nontextured) surfaces was found to be $2.9 \pm 0.95\%$ ($n = 19$ cultures). A surface with a series of $10 \mu\text{m}$ wide grooves separated by $10 \mu\text{m}$ wide ridges and a depth of $5 \mu\text{m}$ ($10 \times 10 \times 5$) yielded myocytes that were significantly more aligned ($46.9 \pm 4.3\%$, $n = 5$, Fig. 4)

than their flat counterparts ($p < 0.001$). Myocytes plated on a microgrooved membrane with a $10 \mu\text{m}$ groove and a $10 \mu\text{m}$ ridge, but a depth of $2 \mu\text{m}$ ($10 \times 10 \times 2$) yielded myocytes that were significantly more aligned ($69.8 \pm 2.0\%$, $n = 12$) than the $10 \times 10 \times 2$ groove ($p < 0.01$). Decrease of either ridge width ($10 \times 5 \times 5$) or

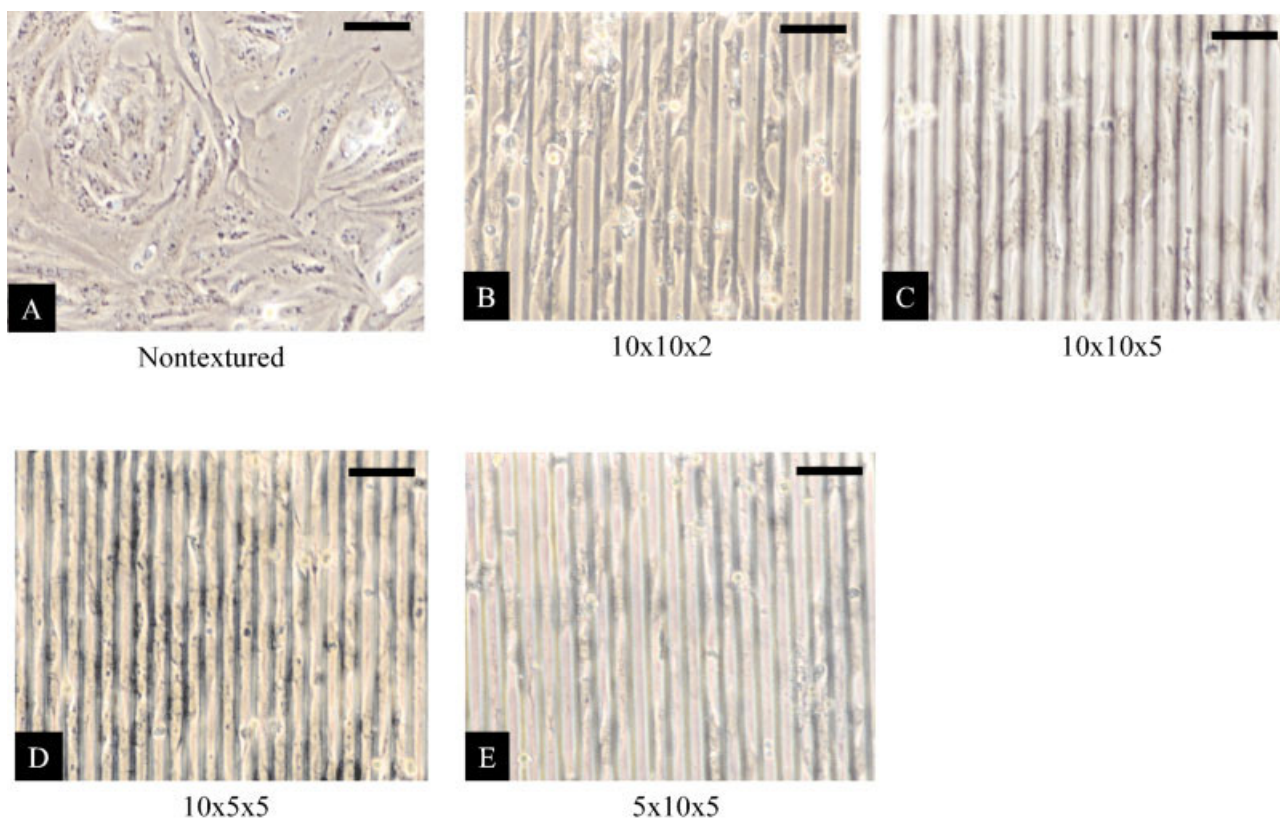


Figure 3. Phase images of myocytes isolated from neonatal rat hearts and plated for 4 days on various surfaces: (A) nontextured; (B) $10 \times 10 \times 2$; (C) $10 \times 10 \times 5$; (D) $10 \times 5 \times 5$; (E) $5 \times 10 \times 5$. Scale bar, $50 \mu\text{m}$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

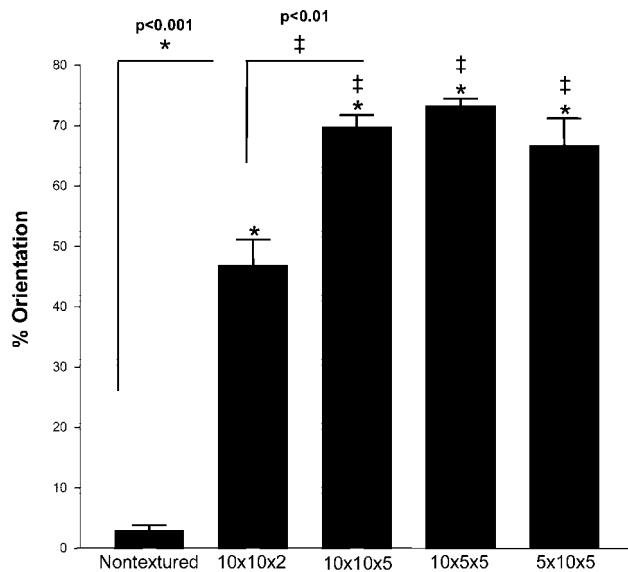


Figure 4. The percent of cell orientation of myocytes on nontextured and microgrooved silicone surfaces was measured as described using the cell edge. Statistical analysis of the data assessed by one-way ANOVA with a Newman-Keuls multiple comparison test.

groove width ($5 \times 10 \times 5$) resulted in oriented myocytes ($73.3 \pm 1.2\%$, $n = 7$ and $66.7 \pm 4.5\%$, $n = 5$; respectively), which were greater than the shallow groove ($p < 0.01$) but not different from the other $5\text{-}\mu\text{m}$ deep microgrooved surfaces ($p > 0.05$).

Localization and expression of connexin43

Connexin43 was tagged using immunocytochemistry and visualized by confocal microscopy in cultured cardiac myocytes plated on various microgrooved substrata as well as the intact neonatal ventricle [Fig. 5(A–F)]. Myocytes plated on nontextured surfaces make contacts indiscriminately with neighboring cells. The resulting formation of gap junctions as examined by connexin43 localization [Fig. 5(A)] follows the irregular outline of the cell. Shallow microgrooves [Fig. 5(B)] yielded myocytes with junctions that were less random than those on the nontextured surfaces but were not as ordered as those in the deeper microgrooves [Fig. 5(C–E)]. Shallow grooves made it possible for the myocytes to spread out of one groove toward another, as seen in Figure 2(B) and more clearly in Figure 5(B). A $5\text{-}\mu\text{m}$ depth coupled with a $10\text{-}\mu\text{m}$ ridge appeared to prevent the myocytes from being able to transverse between grooves [Fig. 5(C,E)]. The myocytes plated on the $10 \times 5 \times 5$ microgrooved surface [Fig. 5(D)], on the other hand, were able to bridge between grooves due to the narrower, $5\text{-}\mu\text{m}$ ridge separating the grooves, thereby allowing myocytes to make the necessary end-to-end connections

with myocytes within the same groove as well as lateral contacts between grooves. The resulting localization of connexin43 in the myocytes plated on $10 \times 5 \times 5$ microgrooved substrata more closely resembled that found in the neonate [Fig. 5(F)].

These observations on junctions were confirmed through the use of Western blot analysis (Fig. 6), which revealed an increase in the protein expression of connexin43 in myocytes plated on nontextured and shallow microgrooved ($10 \times 10 \times 2$) surfaces compared with the neonatal heart ($p < 0.001$, $n = 6$ cultures of approximately 40 hearts per culture). An increase in groove depth to $5\text{-}\mu\text{m}$ ($10 \times 10 \times 5$), regardless of changes in groove width ($5 \times 10 \times 5$) or ridge width ($10 \times 5 \times 5$), resulted in connexin43 expression recapitulating that of the neonate ($p > 0.05$, $n = 5$ hearts, all from separate litters).

Synchronous beating

Our study examined how slight variations in separation (5 or $10\text{-}\mu\text{m}$) affect the synchronicity of cells that are both connected and aligned. Analysis shows that synchronicity of densely plated cultures does not vary significantly among our surface types, occurring in $96\text{--}99\%$ ($n = 4$) of cells plated on $10 \times 10 \times 2$, $10 \times 10 \times 5$, and $10 \times 5 \times 5$ patterned surfaces, as well as on flat membranes. Myocytes on $5 \times 10 \times 5$ patterned surfaces were too close to resolve and were therefore not included in the analysis.

Localization and expression of N-cadherin

N-cadherin is localized to cell surfaces in myocytes plated on microgrooved substrata as well as in the neonate [Fig. 7(A–F)]. On nontextured surfaces [Fig. 7(A)], N-cadherin is found around the perimeter of the myocyte as it makes random contacts with neighboring cells. The shallow microgrooved surface ($10 \times 10 \times 2$) provided the opportunity to form unsystematic contacts, as visualized through the staining of N-cadherin, between myocytes [Fig. 7(B)] that were similar to that of the nontextured surface. An increase in groove depth to $5\text{-}\mu\text{m}$ [Fig. 7(C–E)] yielded more deliberate patterns of N-cadherin expression between myocytes within grooves. However, the localization of N-cadherin on $10 \times 5 \times 5$ microgrooved surfaces [Fig. 7(D)] most closely resembled that found in the neonate in that the myocytes were adjoined at the ends and made side-to-side connections between grooves as seen in the heart [Fig. 7(F)].

Western blot analysis reinforced these findings with slightly increased N-cadherin protein expression in

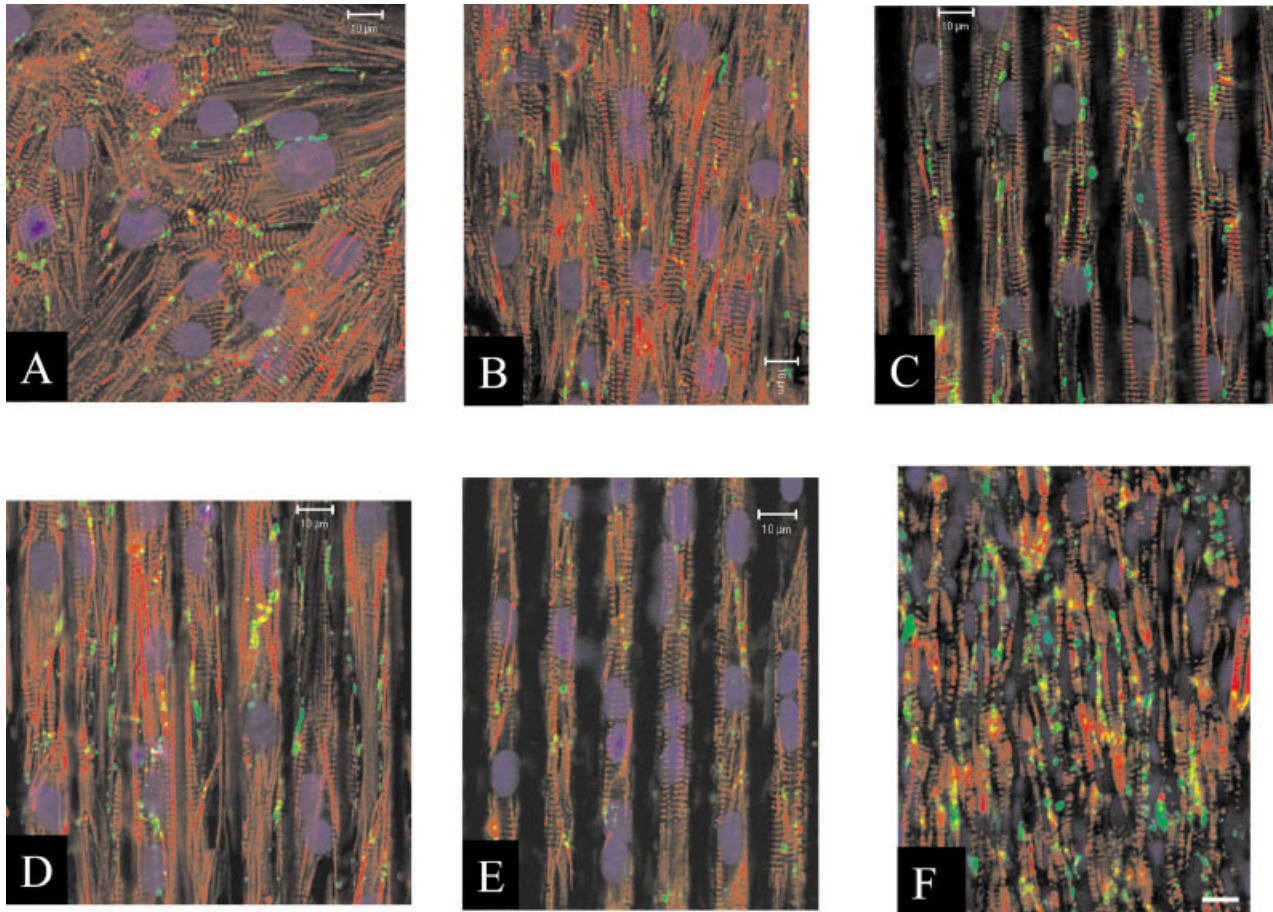
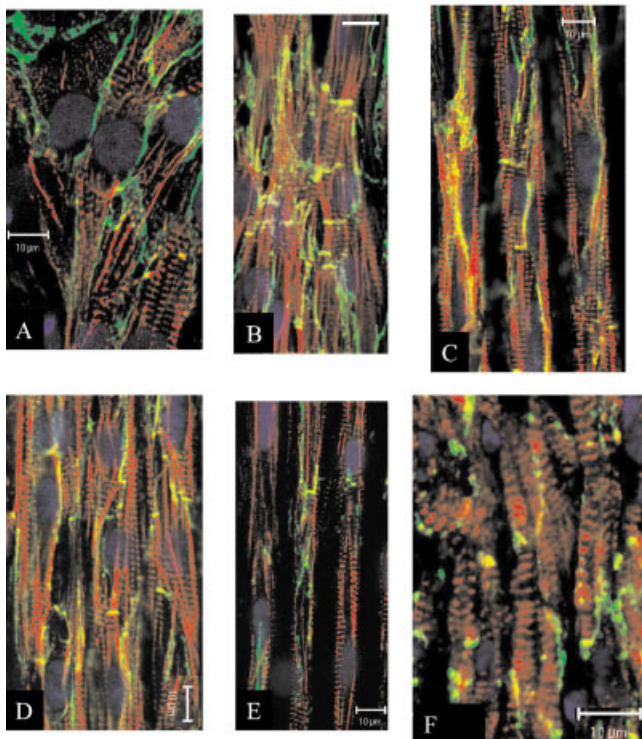


Figure 5. The gap junction protein, connexin43 (green), actin filaments (red), and nuclei (blue) are stained using immunofluorescence. Micrographs show (A) nontextured; (B) $10 \times 10 \times 2$; (C) $10 \times 10 \times 5$; (D) $10 \times 5 \times 5$; (E) $5 \times 10 \times 5$; and (F) neonate. Myofibril alignment and gap junction localization in $10 \times 5 \times 5$ in (D) most closely resemble the neonate (F). Scale bar, $10 \mu\text{m}$.



myocytes plated on nontextured surfaces relative to that in the neonatal heart (Fig. 8). Myocytes plated on shallow grooved surfaces ($10 \times 10 \times 2$) were more aligned than their nontextured counterparts; however, the $2\text{-}\mu\text{m}$ depth of the grooves is not sufficient to curb the levels of N-cadherin expression. An increase in groove depth to $5 \mu\text{m}$ ($10 \times 10 \times 5$) altered the localization of N-cadherin [Fig. 7(C)] but was ineffective in decreasing the total levels of expression (Fig. 8). However, when the width of the ridge separating the grooves was decreased from 10 to $5 \mu\text{m}$ ($10 \times 5 \times 5$), the expression of N-cadherin was significantly less than that found in myocytes plated on nontextured, $10 \times 10 \times 2$ or $10 \times 10 \times 5$ grooved substrata ($p < 0.05$, $n = 6$ cultures of approximately 40 hearts per culture)

Figure 7. The adherens junction protein, N-cadherin (green), actin filaments (red) and nuclei (blue) are stained using immunofluorescence. Micrographs show (A) nontextured; (B) $10 \times 10 \times 2$; (C) $10 \times 10 \times 5$; (D) $10 \times 5 \times 5$; (E) $5 \times 10 \times 5$; and (F) neonate. Myofibril organization and N-cadherin localization in $10 \times 5 \times 5$ (D) most closely resemble the neonate (F). Scale bar, $10 \mu\text{m}$.

but not different from the neonate ($p > 0.05$) when compared using a one-way ANOVA with a Newman-Keuls multiple comparison test. A decrease in groove width from 10 to 5 μm ($5 \times 10 \times 5$) resulted in a significant decrease in N-cadherin expression relative to the neonatal heart ($p < 0.05$) as well as all other surfaces discussed above ($p < 0.05$) using the same statistical analysis.

DISCUSSION

We have optimized the groove dimensions to align neonatal rat cardiac myocytes in a manner that recapitulates their anatomy and physiology *in vivo*. The optimal surface was determined by examining the following parameters in nontextured and microgrooved substrata: the degree of cell orientation, junctional protein localization, expression level of connexin43 and N-cadherin, and the synchronicity of beating.

Many tissues in the body have oriented cells, such as nerve and muscle and it has been previously reported that microfabricated grooved surfaces align different cell types.^{15–18} Orientation is particularly important in the heart where the myocytes transmit force in a directed manner. This essential feature has been recognized in heart research and attempts to produce aligned myocyte cultures *in vitro* have been made. For example, myocytes were oriented by streaking type I

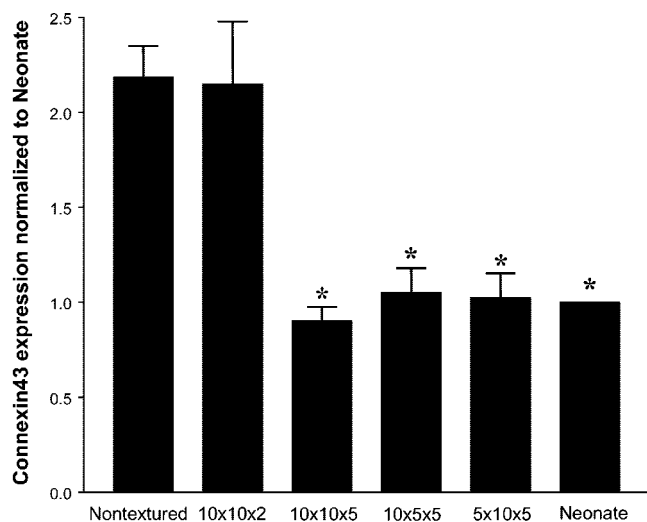


Figure 6. Levels of connexin43 protein expression were measured by Western blot analysis and normalized to the neonate. Connexin43 is over expressed in the nontextured and $10 \times 10 \times 2$ surfaces relative to the neonate ($p < 0.001$, $N = 6$); however, an increase in groove depth to 5 μm ($10 \times 10 \times 5$, $10 \times 5 \times 5$ and $5 \times 10 \times 5$) restores the normal levels found in the neonate. Statistical analysis of the data measured by one-way ANOVA with a Newman-Keuls multiple comparison test. Error bar, SEM.

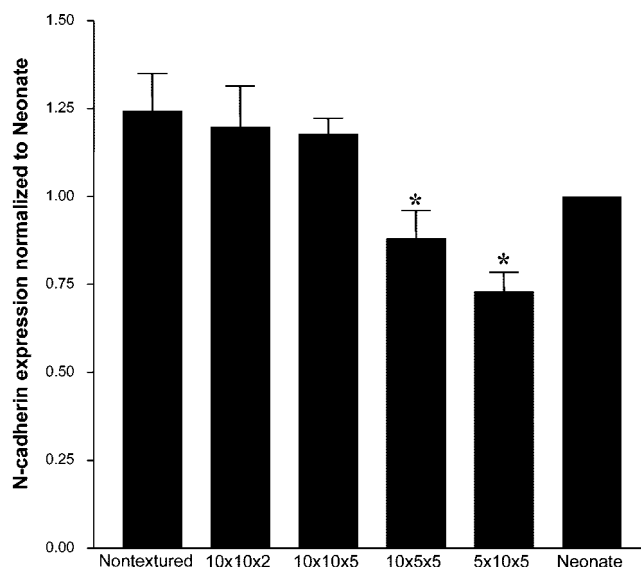


Figure 8. Levels of N-cadherin protein expression were measured by Western blot analysis of approximately 240 hearts from 6 independent cultures and normalized to the neonate. N-cadherin expression on the $5 \times 10 \times 5$ surface is significantly less than the neonate ($p < 0.05$). There is a slight increase in N-cadherin protein expression in the nontextured, $10 \times 10 \times 2$ and $10 \times 10 \times 5$ surfaces, but the $10 \times 5 \times 5$ surface shows a significant decrease ($p < 0.05$), restoring the normal levels found in the neonate. Statistical analysis of the data measured by one-way ANOVA with a Newman-Keuls multiple comparison test. Error bars, SEM.

collagen in parallel arrays on glass coverslips¹⁹ or on silicone membranes,²⁰ which were then subjected to longitudinal or transverse loading with the use of a static stretching device.²¹ However, alignment of collagen can be patchy, limiting analysis to anatomical studies, and eliminating molecular or biochemical analysis of the whole dish. Also, the collagen was not covalently bound to the surface and could, therefore, detach from the silicone surface upon a quick stretch, making delivery of dynamic or cyclic mechanical stimulation ineffective. Bursac et al.²² have recently shown that microabraded coverslips yield cell elongation and coaligned cells. Other methods for alignment include micropatterning via microcontact printing^{22,23} or soft lithography.²⁴ Although these methods are reliable and effective in yielding oriented cell cultures, the approach is limited in that protein patterns lose their integrity over time,²⁵ and cells are exposed only to a two-dimensional, bottom surface. The lanes must be microstamped sufficiently far apart to prevent cells from migrating between them, because this would abolish the alignment. Additionally, these laterally displaced lanes result in aligned myocytes in columns that do not connect to each other. This interplay between lane spacing and alignment is not an issue with photolithography because this method forms a three-dimensional structure into the surface, providing greater control over the x , y , and z dimensions.

Alignment, 3D topography, and lateral spacings are essential for the precise expression of cell junctional proteins and their localization. Gap junctions provide a low-resistance pathway by which electrical signals can be propagated between cells, allowing cardiac muscle to function as a synchronously beating unit.⁷ It is, therefore, important to note that our new culture environment permits development of life-like contacts where myocytes communicate normally by electrical and mechanical junctions. These types of connections are absent or compromised in isolated adult myocytes, cardiac cultures plated at low cell densities, and most high-density cultures as well. Many cell processes are influenced by cell–cell contact; for example, adrenergic receptor-mediated responses have been shown to be determined by the amount of cell contact.²⁶

We find that myocytes on flat, nontextured surfaces, overexpress connexin43 relative to that found in the neonate heart. Despite the increase in alignment of a shallow groove compared with flat, nontextured surfaces, the protein expression of connexin43 is still higher than that found in the neonate. It is an increase of groove depth to 5 μm that brings connexin43 expression back to levels present in the neonate. N-cadherin expression is somewhat sensitive to lateral spacings (within the range of 5–10 μm) and requires a 5 μm ridge or groove to recapitulate the protein expression levels and localization of the neonatal heart. Perhaps topography mimics a remodeling process that occurs with the heart's maturation. Connexin43 expression increases sharply during embryonic stages and then falls during postnatal development.²⁷ This would suggest that myocytes plated on nontextured and even shallow microgrooved surfaces have reverted back to connexin43 levels of earlier stages of development; whereas the myocytes on microgrooved surfaces with a depth of 5 μm match the connexin43 expression of the neonatal heart.

Previous studies examining synchronicity of aligned myocytes have shown the degree of separation between myocytes affects both their electrical connectivity and consequently the uniformity with which they contract.²³ However, the dimensional differences examined were such that adjacent aligned myocytes were either very close (10 μm), and hence able to make electrical connections, or extremely far apart (80 μm) and unable to connect. Our study examined how smaller variations in separation (5 or 10 μm) affect the synchronicity of cells that are both electrically connected and aligned. Despite differences in both expression level and localization of connexin43, any change in synchronicity was masked in high density where most of the myocytes are electrically connected. Taking together our data on synchronicity, anatomical localization, and levels of protein expression, the pattern of connectivity that exists *in vivo* is best replicated by the 10 \times 5 \times 5 surface.

Mechanical stresses impact myocytes *in vitro* causing activation of many intracellular signals, which lead to hypertrophy.²⁸ The study of such responses is important in understanding the underlying principles of heart failure. Microgrooved substrata would be an ideal environment for such studies. Once aligned, cells may be examined under a static longitudinal or transverse strain or a dynamic uniaxial strain system.²⁹ Chronic (6 days) unidirectional stretch has been shown to align myocytes along the direction of force but it also causes hypertrophy.³⁰ Linear pulsatile or static stretch delivered to a monolayer of randomly oriented myocytes has been reported to induce up-regulation in connexin43 and N-cadherin expression along with an increase in conduction velocity, as early as one hour after stretch.³¹ Studies such as these have provided insight into how the myocyte responds to stretch; however, since our microgrooved surface is superior it would permit better experiments to be done.

An optimized microgrooved substratum that reintroduces more physiological microarchitecture to a tissue culture system has many potential uses in drug screenings and diagnostic applications. The substratum can also be readily changed to a biodegradable polymer, which would be very useful in devising constructs for cardiac tissue engineering applications. Overall, we conclude that the most optimal topography for neonatal cardiac myocyte cultures was a microgrooved surface with a 10 μm groove, 5 μm ridge, and 5 μm depth. This permitted normal anatomical, molecular, and physiological function in the culture as expected for a neonatal heart in the rat.

The authors thank Mr. Samuel Senyo for fabricating the parylene templates and Ms. Sara Motlagh for assistance in quantifying protein expression.

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