

LINEAR FIBROBLAST ALIGNMENT ON SINUSOIDAL WAVE MICROPATTERNS

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ABSTRACT

We investigated the behavior of mouse embryonic fibroblasts on *non-linear*, sinusoidal wave grooves created via electron beam lithography (EBL) on a polymethyl methacrylate (PMMA) substrate. Three different wave patterns, with varying wavelengths and amplitudes, and two linear line patterns were created. For all wave patterns, we noted that cells did not reside within grooves as in line patterns; rather they aligned in a linear fashion, contacting the inside and outside of grooves. 74-81% of cells were aligned on the wave patterns, while 88-95% of cells were aligned on the line patterns.

KEYWORDS

Contact guidance, fibroblasts (3T3), micro patterning, polymethyl methacrylate (PMMA), cell alignment, micro topography, electron beam lithography

INTRODUCTION

Mammalian cells *in vivo* exist in a complex environment, which provides an intricate set of signals that affect cell behavior. Of these signals, there are naturally occurring micro- and nano-topographical cues within the extracellular matrix which can influence cell arrangement, migration, and orientation [1]. A generic goal of tissue engineering is to create materials that may regulate and emulate spatial cues. As new implant materials are developed, understanding cell-substrate interactions has become increasingly important. For many years, it has been recognized that cells respond to underlying substratum topographical features [2]. This phenomenon is termed contact guidance and is characterized by cell response to micrometer and submicrometer surface features. It has become clear that nearly all cell types will react to surface topography through adhesion, spreading, migration, and/or proliferation [3]. To date, this behavior has been studied predominantly on linear grooved patterns created through micro-machining, photolithography, and electron beam lithography. Linear groove patterns typically result in cell elongation, migration guidance along the direction of the grooves, and reorganization of the cytoskeleton [4]. Micro- and nano-pillars and micro- and nano-wells have been found to affect cell adhesion to surface substrata by either increasing or decreasing cell attachment depending on spacing and feature size [5]. Studies on hexagonal and random patterning have also taken place [6], but the majority of contact guidance has involved linear grooves or rectilinear arrays of posts and wells. However, in nature curvilinear architecture exists in many tissues and organs. This is vital for organ geometry and function. In this study, we investigate the behavior of mouse embryonic fibroblasts, on curvilinear, sinusoidal wave grooves created using electron-beam lithography (EBL) on a polymethyl methacrylate (PMMA) substrate.

MATERIALS AND METHODS

Pattern Fabrication: A diced positively charged microscope slide was spin-coated with PMMA and subjected to EBL, using a FEI Inspec scanning electron microscope equipped with a nanopattern generation system to etch line and wave patterns created using CAD software. Wave patterns were defined by their amplitude (A) and wavelength (λ) in μm (wave 1: A=40, λ =10; wave 2: A=30, λ =5; wave 3: A=30, λ =10); all wave patterns had groove spacing of 20 μm . Linear patterns were used as a positive control for alignment; they were defined by their line spacing, 20 μm or 10 μm . After fabrication, patterns were measured and inspected using a Veeco Dimention 3100 AFM.

Cell Culture: NIH 3T3 mouse fibroblasts were grown in Dulbecco's Modification of Eagle's Medium supplemented with 10% newborn calf serum, 1 M HEPES, 1% antibiotic, and 2% L-glutamine. Cells at 80-90% confluence were detached from the culture flask surface using trypsin and collected via centrifugation. Cells were resuspended to yield a final concentration of 200,000 cells/ml. 200 μl of the cell suspension was placed on the surface of the chips and allowed to seed for 10 minutes, after which an appropriate amount of media was added to the culture dish. The cells were incubated in 95% air, 5% CO₂ at 37°C for 4, 24, or 48 hours before staining. Cells were stained for the visualization of actin filaments, using TRITC-conjugated phalloidin, and cell nuclei, using fluoroshield with DAPI.

Image Analysis: An inverted epi-fluorescence Nikon microscope was used for imaging stained cells using 10x and 60x objectives. Cell number, length, and width data was collected. Images were analyzed using ImageJ. For orientation data, pattern direction was designated 0°. Cells were considered aligned if they were within $\pm 15^\circ$ from the pattern direction. For control surfaces, the y-axis of the image was used as 0°.

RESULTS AND DISCUSSION

AFM imaging revealed that EBL created well-defined sinusoidal and linear grooves in the PMMA substrates, Figure 1. The PMMA thickness was between 650 and 680 nm, and groove width was between 4.6 and 5 μm in all patterns. PMMA was etched completely in areas exposed to the electron beam, thus, exposing the underlying positively charged glass.

Cells were cultured 4, 24, and 48 hours on the patterned surfaces and an unpattern PMMA surface as a control, stained, and imaged. Clear fibroblast alignment along wave and linear patterns was noted along PMMA patterned surfaces, seen in Figure 2. Fibroblasts on control surface tended to be randomly oriented and spread out, whereas cells on the patterned surfaces tended to be elongated and oriented with the pattern. Interestingly, alignment between wave and line patterns was similar despite the distinct difference in pattern shape. Table 1 shows percent cell alignment under static conditions for fibroblasts at 4, 24, and 48 hours after initial cell seeding. After 4 hours, alignment was seen on the line patterns, as 88% and 95% cells aligned, with wave patterns at 74%, 81%, and 78% aligned, compared to 14% of the unpatterned control cells aligned. The majority of cells were seen to orient along the pattern direction for both wave and line patterns, with the greatest alignment seen 4 hours after seeding. Figure 3 shows histogram data for cells after 24-hour cell incubation. There is a clear trend in alignment for all pattern surfaces. For all wave patterns studied, we observed that cells did not reside within the groove, rather they were noted to reside largely on the elevated flat surfaces adjacent to the groove, crossing over the groove, typically aligning in a linear fashion (Figure 2), similar to the alignment on the line patterns. For the linear patterns we observed that cells aligned parallel to the patterns and tended to reside within the grooves (Figure 2), consistent with previous observations [7].

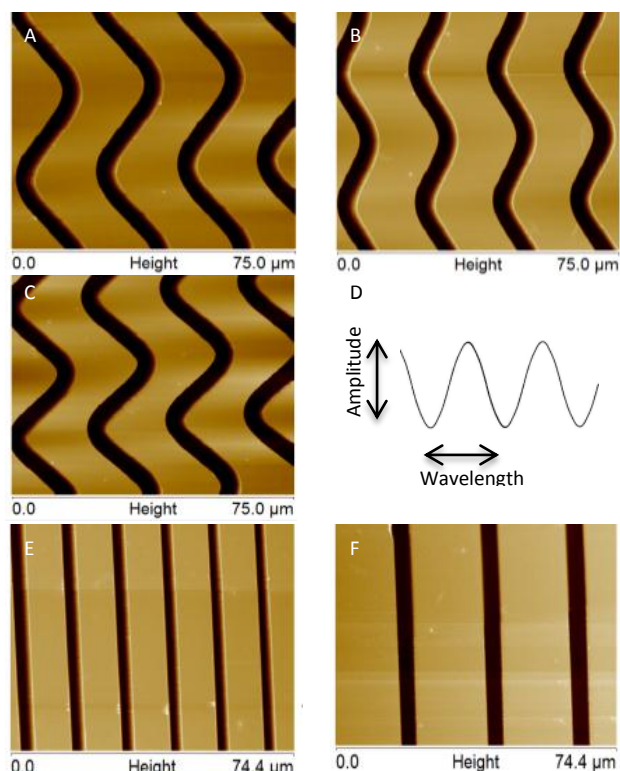


Figure 1. AFM images of patterns on PMMA surface. A: Wave 1, B: Wave 2, C: Wave 3, E: 10 μm spaced lines, F: 20 μm spaced lines.

A z-stacked image was taken using a Nikon confocal microscope to observe differences cell anchoring between line and wave patterns. As can be seen in Figure 4, for line patterns, cells tend to reside inside the groove, i.e. within the trough, (A and B) whereas for wave patterns cells reside both inside *and* outside of the groove (C and D). Actin filaments are stained red. Thus, bright red indicates more actin filaments and black indicates lack of actin. In A, a concentration of actin can be seen along the groove. Since this is the bottom slice of the stack, this indicates that the cell is lying within the groove. This can be confirmed with the top slice, as it shows an absence of actin along the groove. In the wave patterns, we see that cells cross over the grooves and dip into each groove as it passes over it.

Cell adhesion was observed at 24 and 48 hours and compared per pattern type, Figure 5. It was noted that more cells tended to reside on wave pattern 3 and the 20 μm line spaced pattern after 48 hours.

To increase the understanding of cell behavior on artificial surfaces, we have observed cell behavior on non-linear, sinusoidal patterned surfaces. Interestingly, we observed linear cell alignment to the wave patterns and increased cell

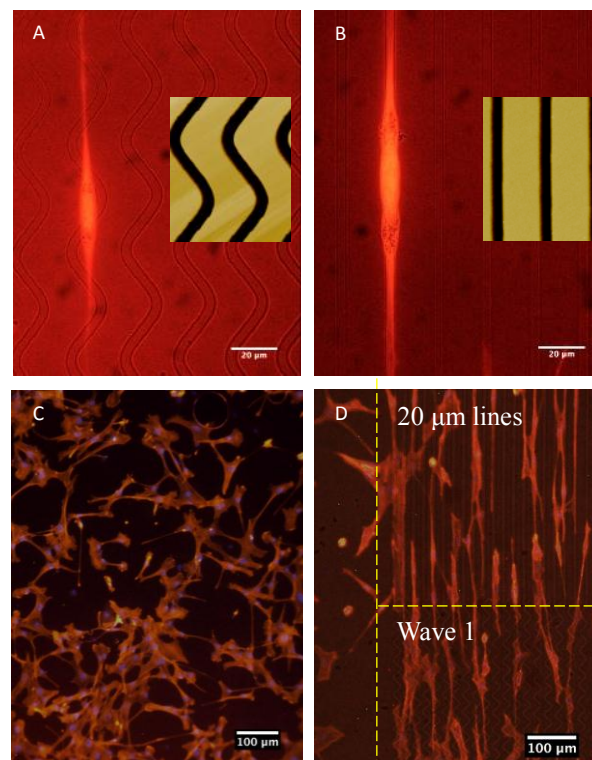


Figure 2. Fibroblast alignment on PMMA. A: Wave 1 pattern, 60x, B: 20 μm spaced lines, 60x, C: control surface (no pattern), 10x. Scale bars are 20 μm for A and B, 100 μm for C.

Table 1. Percent fibroblast alignment per pattern.

Pattern	4 hours	24 hours	48 hours
Wave 1	74%	53%	63%
Wave 2	81%	68%	60%
Wave 3	78%	52%	77%
10 μm lines	88%	74%	83%
20 μm lines	92%	68%	73%
Non-patterned	14%	8%	N/A

adhesion with specific wave dimensions. In the future, we plan to investigate the mechanisms involved in cell adhesion on underlying wave-patterned surfaces and the impact of such attachment on cell function and resistance to detachment under flow conditions.

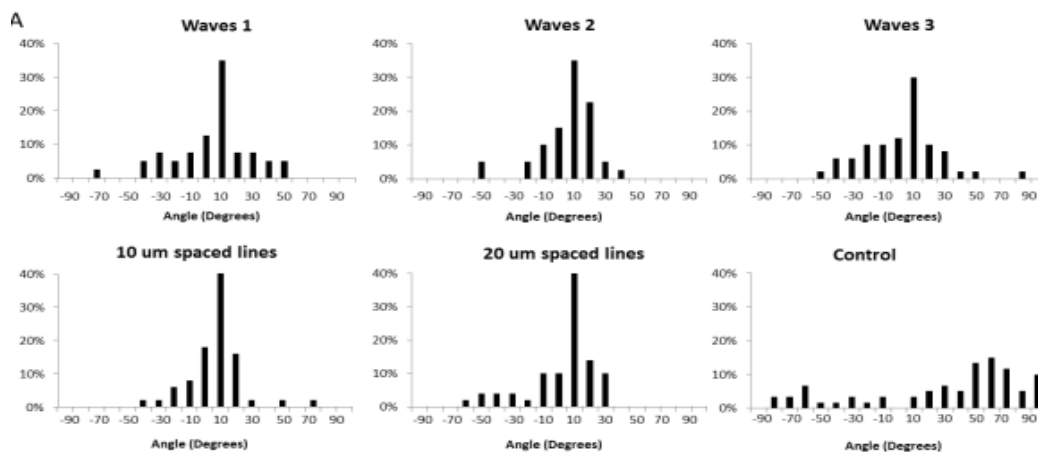


Figure 3. Fibroblast alignment 24 hours after seeding on patterned surface. Frequency on the histograms is plotted as a percentage of total cells counted, between 40-60 cells per pattern type.

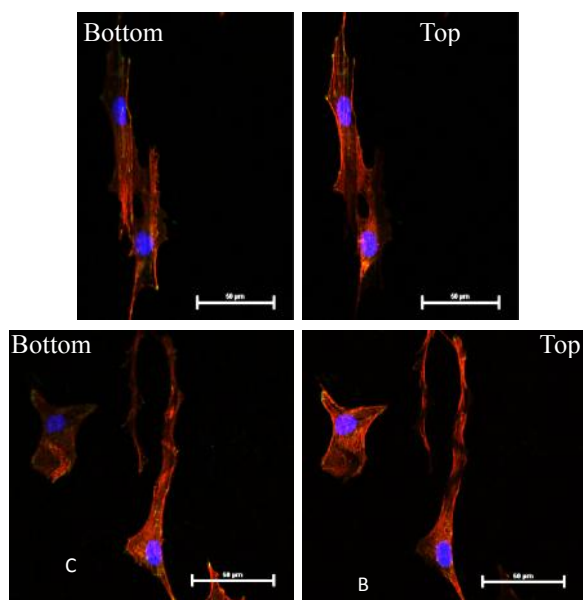


Figure 4. Top and bottom slices from a z-stacked image of cells on A, B: 10 μm spaced line pattern and C, D: Wave 3. Red stain is actin filaments, green is vinculin, and blue is nuclei.

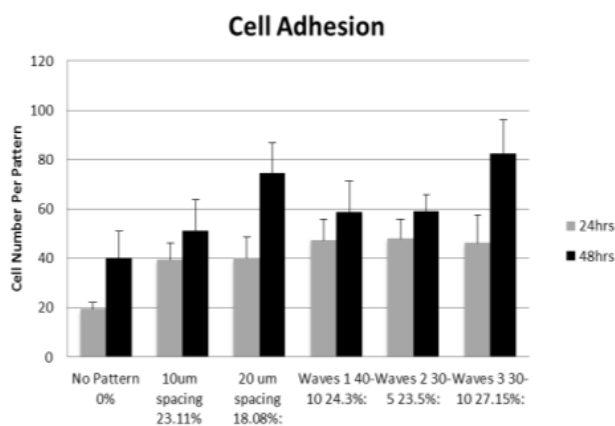


Figure 5. Cell adhesion on each surface tested at 24 and 48 hours. Percentage underneath pattern name displays the amount of glass exposed over total area

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