

Controlling Mammalian Cell Spreading and Cytoskeletal Arrangement with Conveniently Fabricated Continuous Wavy Features on Poly(dimethylsiloxane)

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This paper reports a simple and versatile technique for generating structures on the surfaces of poly(dimethylsiloxane) (PDMS), approximately sinusoidal waves with periods between 0.1 and 10 μm , and the use of these structures to study cell contact guidance.¹ The features are generated by stretching PDMS slabs mechanically, oxidizing them in an oxygen plasma, and allowing them to relax. These surface features are similar to photolithographically fabricated grooves that have traditionally been used to investigate cell contact guidance, although their edges are rounded rather than angular. Bovine capillary endothelial cells align and elongate on these features. The morphology and cytoskeletal structure of the aligned cells are similar to those of cells described in previous studies of contact guidance on surfaces with other types of topography. These observations and comparisons indicate that sharp edges in the features defining the grooves are not essential in eliciting contact guidance. This technique provides a method for fabricating microfeatures for the studies of the interactions between cells and their environment that does not require a cleanroom or access to photolithographic tools.

Introduction

This paper describes an experimentally straightforward technique to fabricate arrays of ordered sinusoidal features on the surface of poly(dimethylsiloxane) (PDMS). It also summarizes the use of these structures to control cell spreading and to investigate the mechanisms employed by the cell to adapt to the topography of the substrate on which it rests. This technique does not require the use of photolithography: it is based on the spontaneous buckling of PDMS whose surface has been oxidized in an air plasma under specific circumstances.² It is possible to fabricate approximately sinusoidal waves with wavelengths between 0.1 and 10 μm ; the corresponding depths of these waves are between 0.01 and 1 μm . These dimensions are well suited for the study of the response of cells to topographical features in vitro, because cells react strongly to features in this range of sizes.¹

The tendency of surface topography to influence cell spreading is called "contact cue guidance".^{1,3–6} Systematic studies of contact cue guidance have used features fabricated photolithographically, sometimes coupled with a microelectromechanical system.^{7,8} Photolithography and associated methods of fabrication have several disadvan-

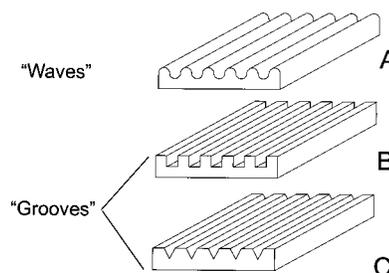


Figure 1. Schematic illustration of the features that we have used (A) and those used historically (B, C). In (A), the ratio of wavelength to amplitude of the waves is approximately 10.

tages, including the high cost and substantial inconvenience of using clean room facilities, the inability to generate gradients in features easily, and the opacity of silicon (the most commonly used material of fabrication) to visible light. The methodology described in this paper overcomes these difficulties: we demonstrate the application of the microfeatures fabricated using this simple technique in cell biology by controlling the spreading of bovine capillary endothelial (BCE) cells with surfaces bearing patterns of sinusoidal waves.

The cross sections of these topographical features are approximately smooth sinusoidal waves; we refer to them as "waves" (Figure 1A). We refer to features whose cross sections are square or V-shaped (both of which have sharp edges) as "grooves" (Figure 1B,C). Grooves with a range of depths and periodicities (from tens of nanometers to hundreds of micrometers) have been the substrates most widely used in studies of contact cue guidance.^{7–14} The

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cylindrical surfaces of optical fibers have also been used in these studies.^{15,16} Continuous sinusoidal wave features comparable in size with the grooves that are commonly used have not been systematically investigated.^{8,17}

The ability to control the topography of surfaces offers one approach to answering certain types of questions in cell biology concerning the influence of topography on cellular behavior. Many hypotheses about the mechanism of cellular reaction to topographical features are centered on the geometrical details of the features that the cells may encounter.¹⁸ For example, some investigators suggest that cells sense edges and require an abrupt change in topography (as observed in grooves or ridges) in order to react to the substrate.¹⁸ The grooves used previously all had sharp edges, and cylinders are not continuous features. It has, therefore, been difficult to study how cells react to continuous topographies that do not present sharp edges. Waves represent a unique topography that is both continuous (like grooves) and have no sharp edges in shape (like cylinders). We have compared cellular reactions to smooth contoured surfaces (e.g., waves) and contoured surfaces that have sharp edges (e.g., grooves) and illustrated approaches to some questions concerning the mechanism of contact guidance. Wave features may also be more relevant to physiological contact cue guidance than angular features, since there are few sharp edges in the cellular environment experienced by cells inside the body.

Results and Discussion

Surface Features. We generated waves with sizes between hundreds of nanometers and tens of micrometers. These dimensions are compatible with studies in cell biology, because individual cells normally have dimensions of tens of micrometers. These structures were fabricated as outlined in Figure 2, route A. This method is simpler than that developed previously (where the PDMS was heated, oxidized, and allowed to cool).² The waves are well ordered over distances of 5 mm and are ideal for studies in cell biology because high perfection and long-range order are seldom required in these studies. Images from both light microscopy (Figure 2, insert I) and scanning electron microscopy (Figure 2, insert II) confirm that the features produced have regular periodicity and amplitude. The dimensions of the waves can be controlled easily by varying the duration of oxidation.² The longer the oxidation, the larger the wavelength and the amplitudes (Figure 3). There is a practical limit, however, to the sizes of waves that can be generated. Because oxidized PDMS is brittle, crack defects that run perpendicular to waves become more common at longer oxidation times (>30 min). This restriction dictates the upper limit of the amplitude and wavelength of these waves at 2 and 20 μm , respectively. Other ways to vary the wavelengths and amplitudes of the waves include controlling the thickness and elastic modulus of the PDMS slab. Thin and soft PDMS slabs tend to produce large waves.²

We also generated gradients of waves using the method described in Figure 2, route B. As in method A, we wrapped a PDMS slab around a cylinder. *In addition*, we placed another PDMS slab in tangential contact with the bent PDMS slab. The flat top slab shields the bent slab from oxidation increasingly as the bent and flat surfaces become closer to each other (from 0% shielding in areas far away from the top slab to 100% shielding on the area of contact between the two PDMS slabs). This procedure gives a gradient of waves whose wavelengths decrease from a maximum of 10 to 0 μm (Figure 2, insert III). The ability to generate a gradient in the dimensions of features with ease is another advantage that this new method has over previously reported techniques.²

The mechanism by which the waves on the surface are generated can be summarized qualitatively as follows (more quantitative analysis can be found elsewhere^{2,19,20}): bending the PDMS slab induces mechanical stress on its surface. When oxidized, the PDMS surface is chemically modified: the exposed Si-CH₃ groups are oxidized to Si-OH groups and form a thin, stiff oxide layer. When released from the cylinder, the bulk PDMS returns approximately to its original shape; this relaxation places the rigid surface oxide under compressive stress. This stress is relieved by buckling into continuous and periodic waves.

This method of generating microfeatures offers several advantages, relative to photolithography, as a methodology: (i) It is more convenient experimentally than photolithography, since it does not require masks or access to clean room facilities. (ii) It generates gradients easily. (iii) It generates features with rounded edges. (iv) It can be applied to PDMS; PDMS is a transparent elastomer having the optical properties needed for microscopy and the mechanical properties needed in studies where cells need to be stretched. The disadvantage of this method is the limited range of sizes and geometries that it produces and the fact that the depth and wavelength cannot be easily controlled independently.

Response of BCE Cells to Surface Waves. BCE cells show clear evidence of contact guidance on a contoured surface of PDMS. Within 2 h of cell attachment to the surface, cells respond to the topography; by 24 h, the cells have adapted fully to these features and display distinct elongated cell morphology. Figure 4A shows a monolayer of cells on wave topography. The cells elongate and spread parallel to the direction of the waves. In contrast, cells on a flat substrate show random orientation and less elongation (Figure 4B). Statistical analysis shows that cells are increasingly guided by the waves as the wavelength and amplitude increase, as indicated by increases in the elongation index and decreases in the average orientation angle (Figure 5B) (see Experimental Section for detailed explanation of the method for quantification). Both elongation and orientation angle level off when the wavelength is above 8 μm . It appears that the waves control the spreading of the cells maximally when the wavelength is above 8 μm for the size range tested.

To investigate some of the ultrastructures of cells spread on surfaces patterned with waves, we used confocal scanning microscopy to examine the complex cytoskeleton on contoured surfaces. Actin stress fibers and microtubules align with the general direction of the contoured surface (Figure 6). Within one cell, there are several levels of organization of the cytoskeleton. For instance, on different planes parallel to the mean plane of the surface, actin

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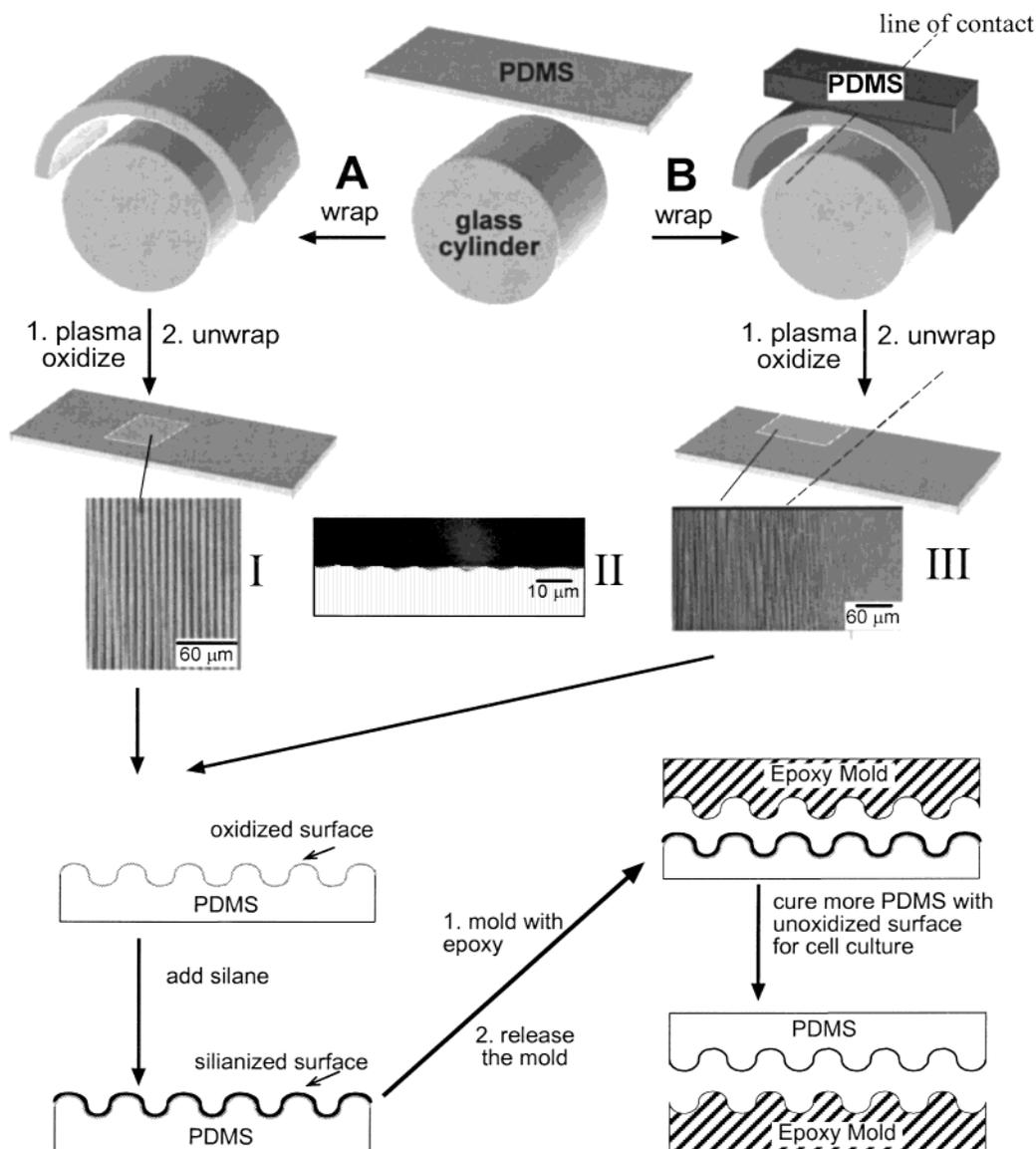


Figure 2. Illustration of the process for generating waves on the surface of PDMS. Top: Route A illustrates the generation of a surface that has waves with uniform pitch. A PDMS slab was first wrapped around a glass cylinder, oxidized in a plasma, and then unwrapped. Waves form in the direction of maximal stress relief. Route B sketches the generation of a surface with a continuous gradient of waves. Inserts I and III are bright field optical micrographs. Insert II is a scanning electron micrograph. Bottom: Once the features are made, the surface is silanized and cast into a UV curable polymer to make the master to fabricate more PDMS substrates. See Experimental Section for details.

fibers organize into quite different macroassemblies. Actin fibers near the substrate (<500 nm) are most strongly guided by the waves; most fibers are straight and extend along the features on the surface (Figure 7A), while actin fibers imaged in a parallel plane $3 \mu\text{m}$ away from the surface (toward the dorsal side of the cell) appear to be more random in orientation (Figure 7B). These findings confirm previous reports on the alignment of actin fibers that many cell types exhibit when cultured on grooved substrates.^{21–23}

We then visualized vinculin to identify focal adhesion complexes (FACs). Although some FACs appear to be colocalized with actin fibers (especially at the termini of

these fibers), there is no general tendency for the FACs to be localized in either the troughs or the crests on the surface features (Figure 8). We infer that, at this stage (36 h after seeding), cells adhere to the substrates conformally, without any preference for adhesion to the troughs or the crests of the wave.

Comparison of Cell Alignment to Waves and Grooves of Similar Sizes. BCE cells clearly respond to surface waves, and this response is manifested both in their morphology and in their cytoskeleton organization. We have compared their responses to waves and grooves and quantified the differences in their reactions to the two different types of substrates. The groove features that we made are illustrated schematically in Figure 1B. The grooves had the same wavelength and amplitudes as the sinusoidal waves fabricated by oxidizing stretched PDMS. Table 1 compares the average orientation angles of BCE cells on two sets of waves and grooves. The results are indistinguishable: the cells do not sense a difference

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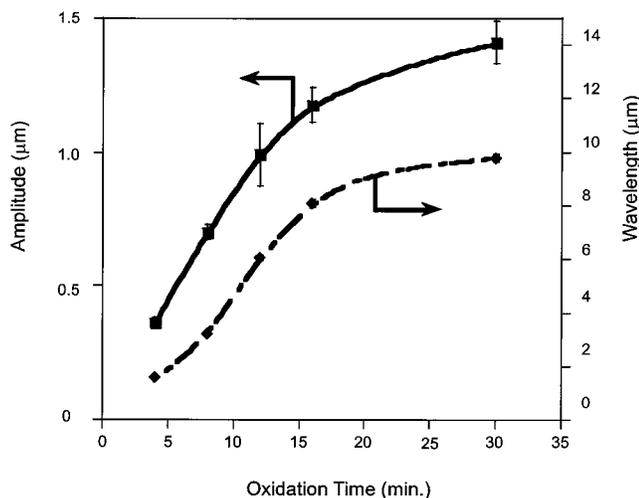


Figure 3. The dependence of wavelengths and amplitude on oxidation time. Increased oxidation time resulted in increased amplitude and wavelength: amplitude, open squares; wavelength, solid diamonds.

Table 1. A Comparison of Cell Orientation on Grooves and Waves^a

feature characterization	wavelength/ μm	orientation angle (deg) (\pm std)
waves	5	20 ± 3
grooves	5	18 ± 3
waves	10	14 ± 4
grooves	10	13 ± 3

^a Orientation angles on wave features are similar to those on grooves.

between the waves and grooves. Actin filaments and microtubule organization of cells on both features are similar to each other. We conclude that cells react to waves in essentially the same way as they do to grooves. To our knowledge, this report is the first one to compare cellular responses to wave and groove features of comparable sizes.

The Mechanism of Contact Guidance. The mechanism of contact guidance of eukaryotic cells is still not well understood.¹ Several hypotheses have been proposed: Ohara and Buck suggested that cellular alignment to grooved surfaces is caused by the alignment of focal adhesion complexes.²⁴ This suggestion was later proven not to be universally applicable for all cases of contact guidance.²⁵ Dunn explained cellular alignment using a model based on the tendency of stress fibers to form in straight lines. Since linear actin fibers cannot form across the ridges and conform to the surface at the same time (as doing so would make the fibers bend up and down on the surface contours), fibers perpendicular to these ridges will not form. Only those fibers parallel with the grooves will tend to form, and thus the stress fibers will force cells to elongate and spread along the direction of the features.²⁶ This theory was later contended by Curtis and Clark, who suggested that cell alignment to grooved surfaces is a result of discontinuities in the shape the surfaces. They observe that actin nucleation sites tend to form at these sharp edges. This observation led them to hypothesize that cytoskeletal polymerization (particularly actin polymerization) tends to occur on or near the sharp edges in the substrate and therefore biases cell spreading to occur along the sharp edges.¹⁸ While hypotheses about the mechanism

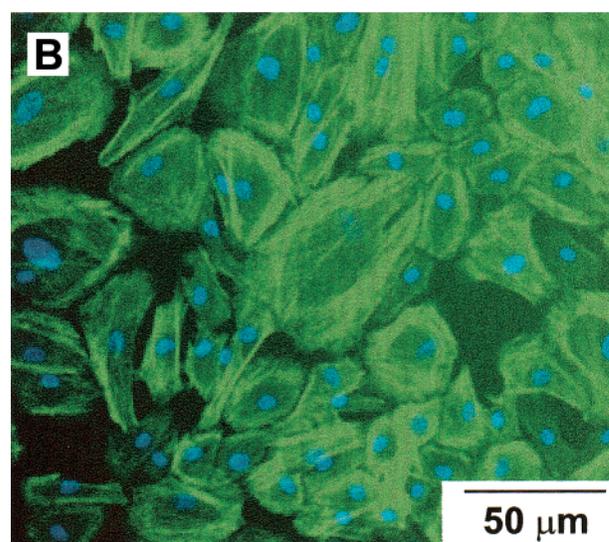
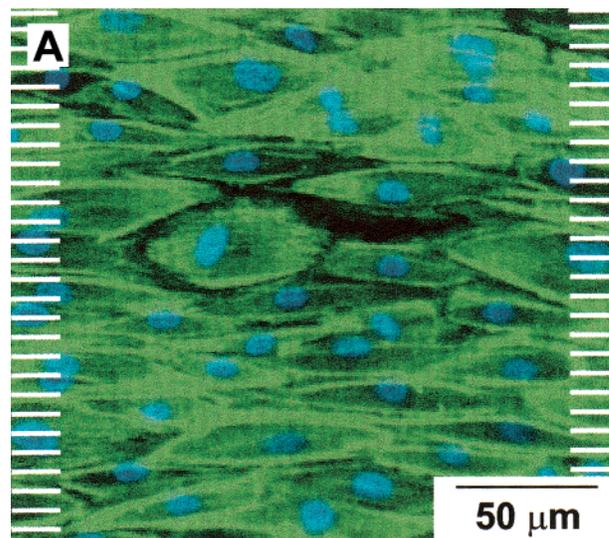


Figure 4. Comparison of cell monolayers on flat and wavy substrates. Cell morphology is visualized using Alexa Fluor 488-conjugated phalloidin that binds to actin filaments with nuclei (blue) stained with 4',6-diamidino-2-phenylindole (DAPI). (A) A confluent layer of BCE cells on a PDMS substrate with features. The grids on both sides of the picture indicate crests and direction of the waves. (B) Confluent layer of cells on a flat PDMS substrate as a control.

of contact guidance abound, none can account for all observations concerning topographical contact guidance. It is clear that the major cellular components responsible for these observations include cytoskeletal elements such as microfilaments and microtubules,²² outside-to-inside signal transducers such as integrins,²⁷ and stress receptors such as chloride channels.²⁸ Microfeatures also affect gene expression and protein synthesis and may influence other parts of the cellular machinery.²⁹ It is not yet clear how the components involved in contact guidance interact and produce the observed cellular response to microfeatured surface.

Our studies provide some additional insights into the factors that determine the behaviors of cells on microstructures. The most significant finding of this study is

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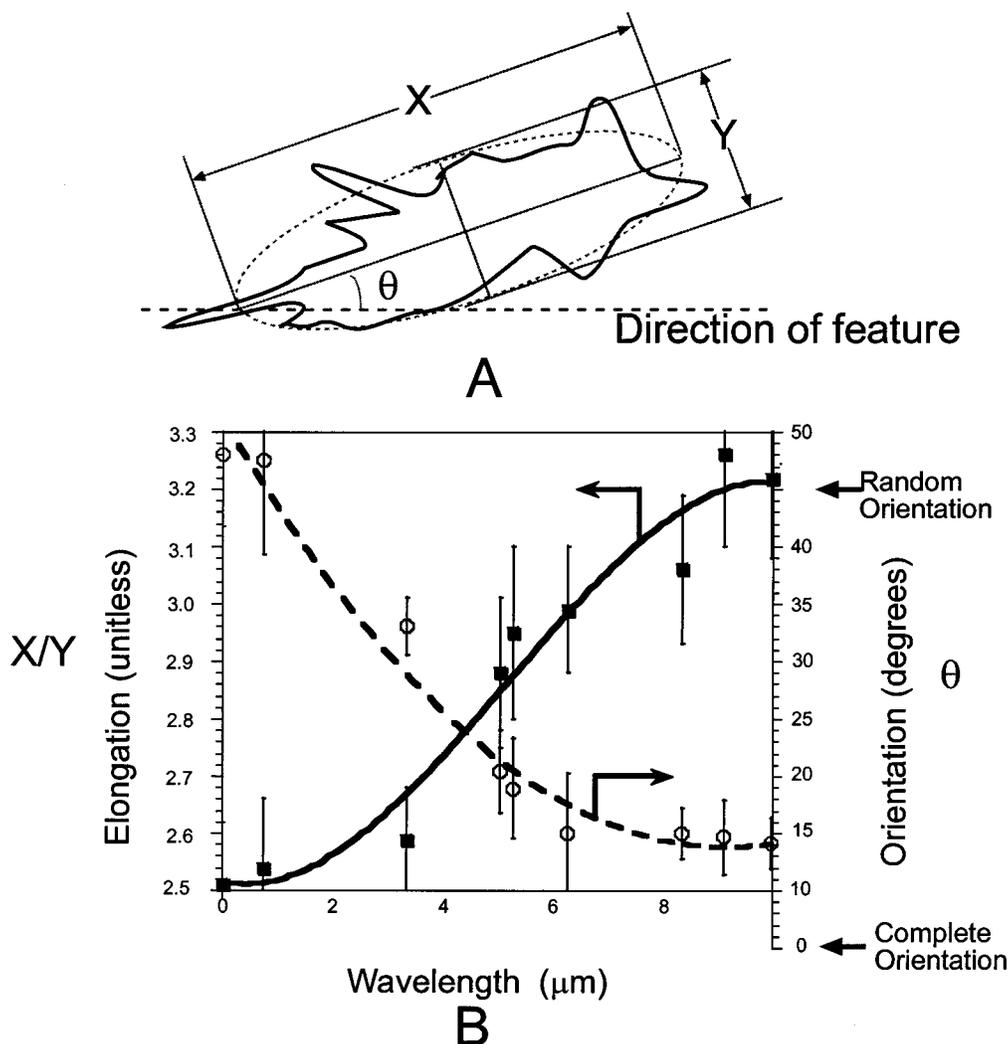


Figure 5. (A) Illustration of the measurement of elongation index, X/Y , and orientation angle, θ . (B) Plots of elongation X/Y (solid squares) and orientation angle θ (open circles) versus the size of the wave features. See text for the methods of measurement. The larger the value of elongation, the more elongated the cell is. The smaller the orientation angle, the more aligned the cell is. Error bars represent one standard deviation from the mean.

the fact that the waves and grooves elicit similar cellular responses. The major topographical factor that distinguishes surface waves and surface grooves is the presence of sharp edges in the latter. Since both waves and grooves control cell spreading in the same way, we conclude that sharp edges are not required to elicit cell contact guidance. This report, we believe, is the first one that uses systematic variation in the fine details of the geometries of the microfeatures to prove that the presence of sharp edges in the microstructure is not a crucial factor in determining cell response to surface topography. Since the mechanisms involved in cellular response to topography are still quite complex, we are, therefore, unable to construct a mechanistic model that accounts for all the observations concerning topographical responses of cells.

Conclusion

We have described a convenient method to fabricate surfaces patterned with ordered arrays of waves to study the interaction between cells and topographical elements. This report is the first to use only wavelike features (continuous and without sharp edges) to control cell spreading on a surface. BCE cells respond to wave and groove topographies in similar ways. We conclude that the sharp edges that characterize grooves are not required to guide cell spreading.

We also believe that the convenience of this technique will find applications in fundamental studies of the interaction between cells and artificial materials and in directing growth of endothelial capillaries in tissue engineering.

Experimental Section

Generation of Desired Microstructures on the Surface. The wave features were fabricated by wrapping a PDMS slab (typically 5 cm \times 3 cm \times 0.5 cm) around a glass cylinder (diameter = 4 cm) and then exposing it to an oxygen plasma for 1–30 min at a pressure between 1 and 2 Torr. After plasma oxidation, the slab was carefully released from the cylinder (Figure 2). To fabricate a master from which additional substrates can be made, we silanized the substrate with (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Bristol, PA) and then cast a UV-activated Epo-Tek polymer (Epoxy Technology, Boston, MA) on the silanized PDMS surface. The hardened polymer was used as the master to produce additional PDMS substrates (Figure 2, bottom). These unoxidized PDMS substrates were used for the studies in cell biology.

The grooved substrates that we used for comparison with the substrates having waves were fabricated by conventional photolithography.³⁰ Briefly, a layer of photoresist (Shipley 1805, Microlithography Chemical Corporation, Newton, MA) was applied to a silicon wafer by spin coating. The thickness of the

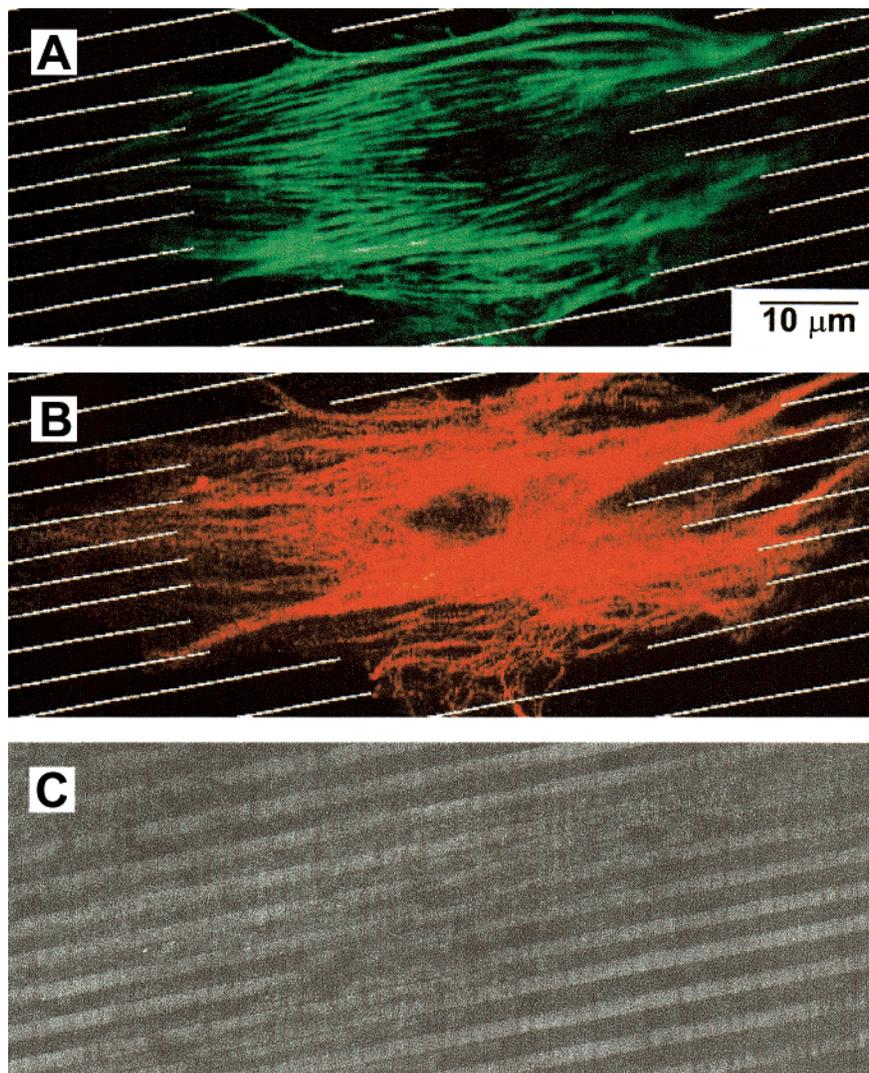


Figure 6. Confocal images of cells on the waves showing actin and microtubule organization. (A) A representative cell whose actin was stained with phalloidin-Alexa Fluor 488 (green). (B) The same cells whose microtubules were stained with Texas red through antibodies. The straight white lines indicate the crests and orientation of the waves beneath the cell. (C) The substrate beneath the cell. This image is collected in the “reflection” mode where all the light is reflected from the sample. Simple difference between brightness does not correctly reflect the heights of the sample. Noise in this image is due to artifacts.

photoresist was controlled by the spin speed. We then exposed the photoresist through photomasks using standard contact-mode photolithography with broadband UV light;³¹ the exposed resist was etched in a KOH-based developer (Microposit 351 developer, Shipley Co., Inc. Marlborough, MA). We then silanized the resulting structure on the silicon wafer with the aforementioned silane in a vacuum desiccator overnight and used it as a master to produce substrates with rectangular grooves by molding PDMS against it.³²

Cell Culture and Attachment to Substrates. Primary bovine capillary endothelial cells (passages 9–12)³³ were grown in Dulbecco’s Modification of Eagle’s Medium (JRH Biosciences, Kansas City, MO) containing 10% bovine calf serum (Hyclone Laboratories, Pittsburgh, PA) and glutamine/penicillin/streptomycin (Irvine Scientific, Santa Ana, CA). In addition, basic fibroblast growth factor (bFGF, Sigma) was present at 5 ng/mL in all media. All cell cultures were maintained at 37 °C in a humidified 10% CO₂ incubator. PDMS substrates were first coated with fibronectin (Sigma, St. Louis, MO, 5 μg/mL in

phosphate buffered saline (PBS)) for an hour in order to allow a uniform layer of fibronectin to adsorb on the PDMS substrates to facilitate cell attachment. Cells were seeded onto fibronectin-coated substrates with a density of 10⁵ cells/mL and allowed to attach and spread for 36 h. The cells then were fixed with 4% paraformaldehyde in PBS for 15 min and subsequently permeabilized with 0.3% Triton-X solution in PBS (5 min).

Immunofluorescence and Microscopy. Actin stress fibers were visualized using phalloidin-Alexa Fluor 488 (Molecular Probes, Eugene, OR). Microtubules and vinculin were visualized by standard immunofluorescence procedures: permeabilized cells were incubated with wash buffer (PBS containing 5% bovine serum albumin) for 1 h at room temperature to inhibit nonspecific adsorption. Primary antibodies raised against vinculin and α-tubulin from mice (Sigma) were applied for 12 h at 4 °C in dilutions of 1:200 in the wash buffer, and the cells were washed extensively with wash buffer. Anti-mouse IgG (from sheep) conjugated with Texas red (Amersham, Newark, NJ) was applied together with phalloidin conjugates, for 1 h at room temperature in dilutions of 1:40 in the wash buffer. Samples were mounted using Vectashield mounting media from Vector Laboratories (Burlingame, CA). Cell nuclei were visualized through 4’,6-diamidino-2-phenylindole (DAPI) contained in the mounting media. Fluorescence images were captured with a Hamamatsu charge coupled device camera mounted on a Leica inverted microscope. Confocal images were obtained using a Leica confocal

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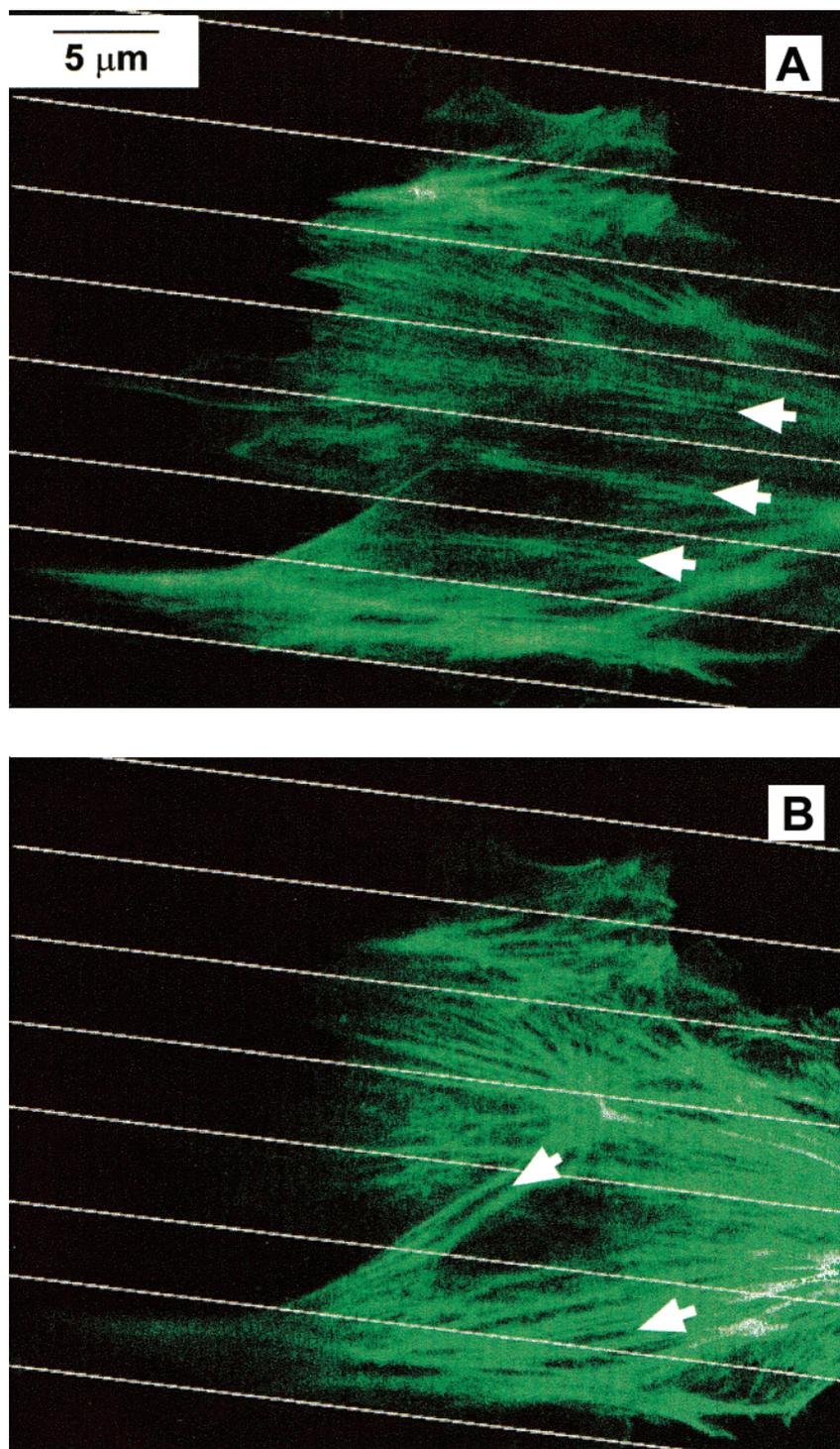


Figure 7. Different levels of actin organization within the same cell attached to an undulating surface. Dotted white lines outline the crests of the waves beneath the cell. (A) Image taken on a plane closest (less than 500 nm from the underlying substrate) to the substrate. Arrows indicate the actin fibers that run parallel to the feature. (B) Image taken 3 μm above the plane of A. Arrows indicate the more random orientation of actin fibers.

laser scanning microscope. The scanning electron microscopy micrographs were obtained from a LEO JSM-6400 scanning electron microscope operating at 1 kV.

Image Analysis. The morphology of the cells on the substrate was analyzed with NIH Image (downloaded from <http://rsb.info.nih.gov/nih-image/>). The projected areas of isolated cells were fit into ellipses (Figure 5A): the long axis of this ellipse was defined as the direction of the cell extension, the perpendicular axis defined the short axis. The ratio between lengths of the long axis (X) and the perpendicular, short axis (Y) was defined as the elongation index. The larger the X/Y ratio, the more a cell is elongated. The angle between the long axis of the ellipse

surrounding the cell and the direction of the feature on the substrate surface, θ , was defined as the orientation angle. On a flat substrate, the angle θ was taken relative to an arbitrary line. A θ of 0° corresponds to complete orientation (i.e., the long axis of the cell is parallel to the crests of the waves) and a θ of 45° corresponds to random orientation. The smaller the angle, the more the cell is oriented with the feature direction. For each particular size of feature, the data reported for elongation and orientation were the averages of data obtained from at least 200 cells. The statistical data were derived both from a set of substrates having different wavelengths and from substrates with a gradient in pitch.

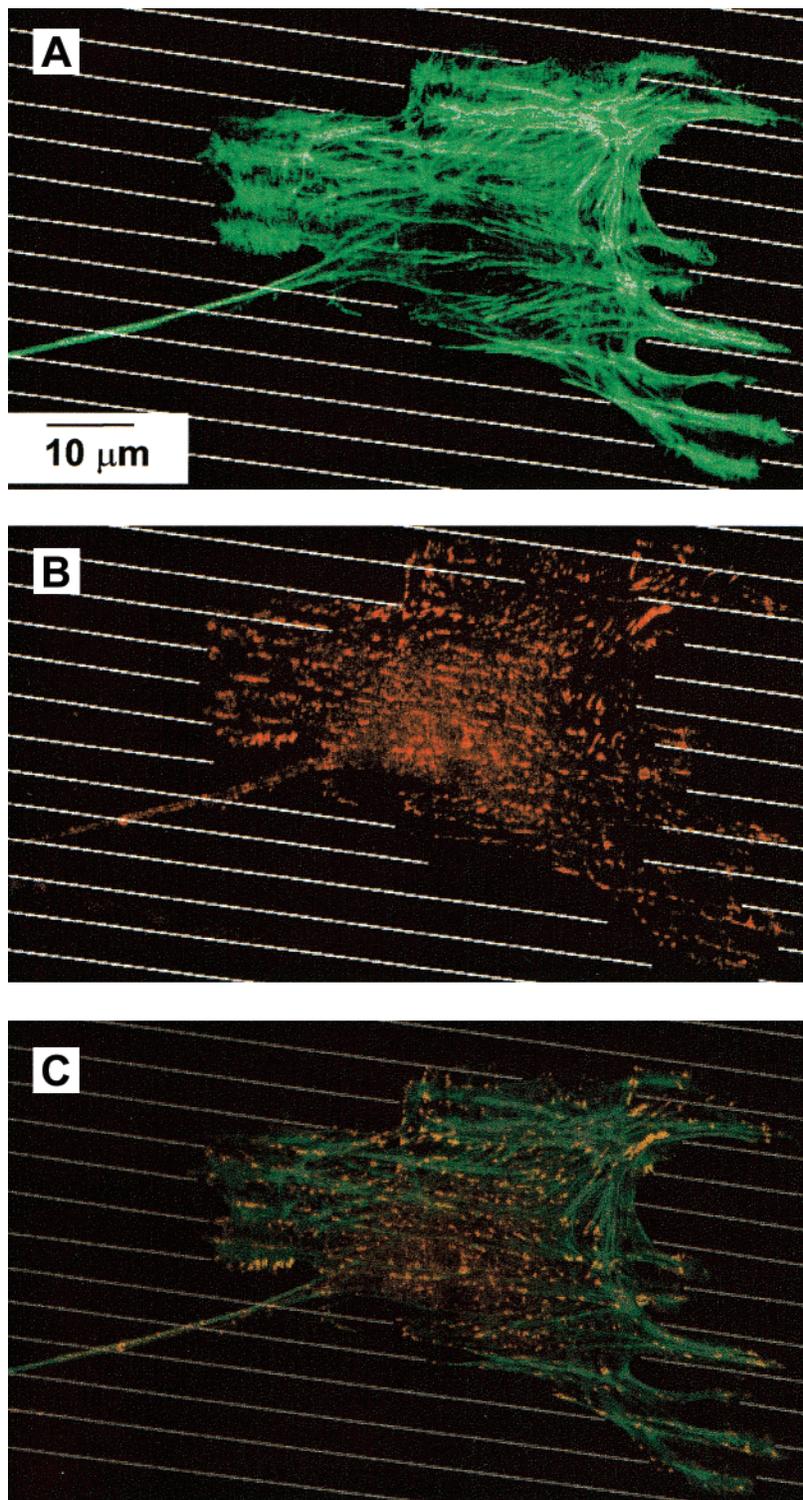


Figure 8. Confocal images of BCE cells on the waves showing actin organization and focal adhesion distribution. (A) One cell whose actin is stained with Alex 488 (green). (B) The same cells whose vinculin is stained with Texas red to visualize focal adhesion. (C) Composite image of A and B. Dotted white lines follow the crests of the waves beneath the cell.

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