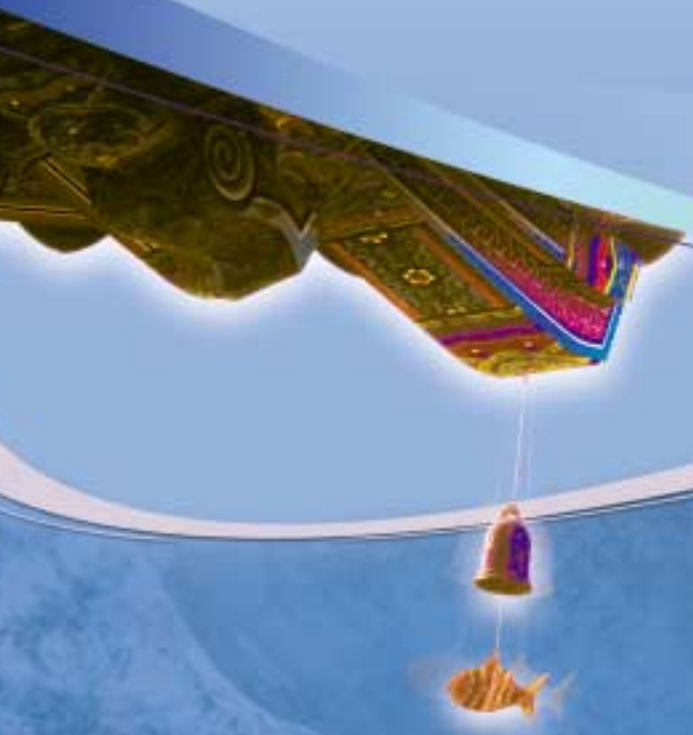
The 7th International Conference on Cellular Engineering

September 6-9, 2005
The Centennial Hall, Yonsei University
Seoul, Korea

from DNA to CLINICS

Program and Abstracts



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THURSDAY 8 SEPTEMBER

09:00-18:00	**Registration**	Lobby, 1F
09:20-10:40	**Podium Session VII** Chairs: Prof. Kang-Yell Choi, Dr. Yeowon Sohn Cell Engineering for Biosensors and Chips & Cell-based Therapy	Concert Hall, Centennial Hall, 1F
S07-1	9:20-9:40 Stem Cell Transplantation Using Umbilical Cord Blood for Ischemic Limb Disease Animal Model and Buerger's Disease Patients *Kyung-Sun Kang (Korea)*	
S07-2	9:40-10:00 Regulation of Neural Differentiation by Neurod2 Protein Transduction *Masayasu Mie (Japan)*	
S07-3	10:00-10:20 Validation of Osteogenic Potential of Human Mesenchymal Cells Using in Situ Quantitative Monitoring System *Motohiro Hirose (Japan)*	
S07-4	10:20-10:40 Cultured Neural Networks: Optimization of Patterned Network Adhesiveness and Characterization of Their Neural Activity *Wim Rutten (The Netherlands)*	
10:40-11:20	**Poster Session II**	Exhibition Hall, Museum, 1F
11:20-12:00	**Plenary IV** Chair: Prof. Suong-Hyu Hyon Some Theoretical Aspects of Cellular and Tissue Engineering *Ivan B. Tokin (Russian Federation)*	Concert Hall, Centennial Hall, 1F
12:00-13:40	**Lunch**	
13:40-14:40	**Podium Session VIII** Chairs: Prof. Jeong-Wook Shin, Prof. Ik-Hwan Kim Cell Engineering for Biosensors and Chips	Concert Hall, Centennial Hall, 1F
S08-1	13:40-14:00 Analysis of Low-Density Neuronal Networks on Micro-Contact Printed Microelectrode Arrays *Sang Beom Jun (Korea)*	
S08-2	14:00-14:20 Ceramic-Based Multi-Parametric Sensorchip for Cost-Effective Monitoring of Living Cells or Tissue *Johann Ressler (Germany)*	
S08-3	14:20-14:40 Guiding Neurons and Fibroblast Cells Using Microfibrication on Photopolymer for In-Situ Navigation of Cellular Patterning *Jae Kyoo Lee (Korea)*	

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GUIDING NEURONS AND FIBROBLAST CELLS USING MICROFIBRICATION ON PHOTOPOLYMER FOR IN-SITU NAVIGATON OF CELLULAR PATTERNING

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Abstract: Cellular patterning has diverse and potential applications in scientific research and engineering. We used laser holographic fabrication to construct the cellular patterning. By the interference of two laser beam on the polymer micro and nanoscale grooves were generated on the photoresponsive polymer. The growth of rat hippocampal neurons were controlled with the microfabricated structure. The directions of neurites were predominant in the perpendicular direction of the grooves and the growth rates of the neurites were also stimulated. Two dimensional cellular patterning of fibroblast cells was constructed along the patterned microstructures on the polymer. These results indicate the feasibility of guiding neurons and fibroblast cells with laser holography, opening the possibility for in-situ navigation in living cells

Introduction

Guiding the growth and attachment of cells to construct cellular patterning is a promising technique in cellular engineering. Various applications have been pursued, including the development of cell-based biosensors, high-throughput screening system, and fundamental studies of cell to cell interactions and cell to material interactions [1]. Especially the neuronal network on small population provides a foundation to investigate the synaptic transmission, neuronal integration, and long-term potentiation [2].

The approaches in cell patterning generally involve immobilizing cell-preferred biochemical to the substrate and fabricating micro and nano structure on the substrate to control the growth and attachment of the cells [3]. Various methods for cell patterning has been developed in last two decades including micro stamping, microfluidic pattering, and stencil pattering [4]. In addition, microfabrication technologies using photolithographic and non-lithographic methods enable the diverse structures for cell substrata such as cliffs, grooves, spikes, tubes, mesh, and random roughness [3].

One disadvantage of these methods are that their cellular patterning is fixed after cell seeding, whereas modifying the cellular structure in a cell culture environment could be used, for example, to change the cell orientation during growth. Controlling the pattering of living cells in real-time involves several approaches, including electric field [5], light [6], electrochemical stimulation [7], and microfabrication [8]. However, These in-situ navigation techniques of cellular patterning exhibit relatively rough guidance in short range. A new technology having the ability to control the cells in more accurate manner extending a larger scale

Azobenzene copolymer is a class of materials having the properties of responding to light. Irradiation of an azo polymer film with two othogoanl laser beams sinusoidal surface relief gratings (SRG) on the surface of the polymer [9]. The interesting and promising application of the azo polymer is in the fabrication of microscale topographies for cell engineering. Optically formed grooves can provide a substrate with a surface topography for controlling cellular growth. Photofabrication with laser holography provides unique advantages over conventional techniques, including being a one-step process without a complicated procedure, and allowing patterning without a photomask, easy control of depth and width, noncontact optical fabrication, and the superimposition and reversibility of patterns [10, 11].

Primary hippocampal neurons have been reported to exhibit a preferred direction of neurite growth perpendicular to microgrooves [12]. Besides, microgrooves influence the orientation and growth of fibroblast cells by contact guidance [13,14]. These reports indicate that the hippocampal neurons and fibroblast cells can be guided by the contact guidance of the groove structure.

In the present study, we inscribed microgrooves on an azobenzene copolymer using laser holography and

observed the behavior of rat hippocampal neuronal cells and NIH3T3 fabroblast cells. The influence of the microstructured surface to cellular growth was investigated with the aim of developing a new method for constructing amd modulating cellular patterning.

Materials and Methods

Photoresponsive polymer: Azobenzene copolymer, poly [(methylmethacrylate) -co-(disperse red 1 acrylate)] (57042-7, Sigma Aldrich) was used as a holographic photoresponsive polymer. It was dissolved in tetrahydrofuran at a concentration of 5% (w/w). The polymer film was formed by spin coating on a cover glass, which produced a coated film with a thickness of about 1 μm. The coated polymer was dried for 6 hours at 70°C to remove the solvent In order to form a holographic SRG.

Surface Relief Grating: In order to form a holographic SRG, we used a 488-nm Ar+ ion laser and the classic Lloyd's mirror setup (Fig. 1). Light from the laser was expanded by a beam expander at an appropriate range and polarized. We formed an interference pattern by the superposition of two beams. One came directly from the laser and the other was reflected from a mirror. The incident beam from the laser was linearly polarized at an angle of +45° while the reflected beam from the mirror had an angle of –45° with respect to the substrate normal. These two orthogonal beams exhibited polarization modulation on the polymer surface resulting in molecular migration to form regular sinusoidal SRG. The intensity of Ar+ ion laser was about 300 mW/cm2. The width of grooves was determined by the combination angle between the

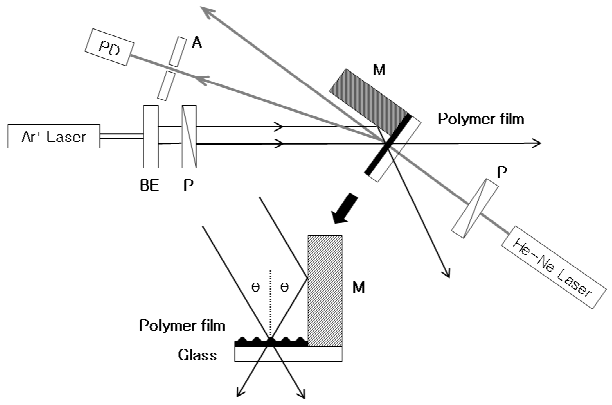

Figure 1: Schematic view of the optic setup for the SRG formation. The laser beam was linearly polarized at an angle of +45° and the reflected beam on the mirror had an angle of –45° with respect to the substrate normal. Two orthogonal beams interfered on the surface of the polymer to form the groove structure. The period of grating pattern depended on the combination angle of Θ. (PD: photo detector, BE: beam expander, P: polarizer, M: mirror, A: aperture)

two beams, and the depth by the duration of laser irradiation. The formation of the grating was monitored in real time by probing with a He-Ne laser (wavelength of 633 nm) onto the inscribed region. The probe beam was linearly polarized at 45° to the axis of grating and did not influence the fabrication process.

Hippocampal Neuron Cell Culture: Primary hippocampal neurons were prepared from embryonic day 18 Sprague-Dawley rats. The dissociated neurons were placed in modified Eagle's medium supplemented with 20% glucose, 1 mM sodium pyruvate, 2 mM L-glutamine, and penicillin-streptomycin (Invitrogen) for 3 hours, and grown in neurobasal medium supplemented with B27 and 0.5 mM L-glutamine. To reduce glial proliferation, we treated the neurons with Ara-C after 3 days. Once a week, we removed half the volume of medium and replaced it with fresh maintenance medium. To sterilize the polymer for culturing neurons, the prepared polymers were dipped into 70% (v/v) ethanol in water and rinsed three times with sterilized water. Prior to cell seeding, cover glasses were treated with poly-D-lysine coating to enhance the adhesion of the neuronal cells on the polymer substrate.

Fibroblast Cell Culture: National Institutes of Health 3T3 fibroblast cells (Korean Cell Line Bank, Korea) were maintained at 37 and 5% CO2 in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 100 units/ml penicillin, 100ug/ml streptomycin and 0.25ug/ml amphotericin B (Gibco). Experiments were performed after the cells were seeded on the photoresponsive polymer at a low density. The fibroblast cells were periodically subcultured in trypsin-EDTA solution containing 0.25% trypsin and 1mM EDTA.

Analysis of Cell Morphology: Differential interference contrast (DIC) optical microscope was used to record the image of the cells with a CCD camera (Axiocam, Carl Zeiss, Germany) attached to a Zeiss LSM microscope. After inscribing SRG on the polymers, the exposed surfaces were investigated with a scanning electron microscope (SEM, XL30FEG, Philips) and an atomic force microscope (AFM, XE-150, PSI) in contact mode. Cell morphology was analyzed with a phase- and differential-contrast microscope. Rat hippocampal neurons were washed with phosphate-buffered saline (PBS) and fixed with 4% (v/v) paraformaldehyde for 10 min at room temperature. After fixation, cells were gently washed with PBS and dehydrated in a series of ethanol solutions (70%, 80%, 90%, then 100% (v/v), each for 10 min) to ensure total dehydration. The samples were desiccated overnight, sputtered with gold, and analyzed by SEM. The fixed cells were gently rinsed three times with warm PBS.

Results and Discussions

Characterizations of Photofabricated Polymer: Figure 2 shows the sinusoidal topographic feature fabricated on the surface of azobenzene copolymer by laser holography. A sectional view revealed that the

microfeatures were homogeneous and sinusoidal-shaped. Ripple structures of small scale on the surface of SRGs were generated during the incomplete process of laser fabricating. Once the fabrication process reached the limitation of fabrication time, the ripple feature initiated to stabilize the structures and disappear from the surface of the microgrooves. The SEM analysis revealed an absence of debris on the surface of the polymer after fabricating the SRG. The surface topography was quite homogeneous in the laser-irradiated area, confirming the success of the fabrication technique.

Neuronal Cells Reaction: The morphology of neurons on the surface patterned with grooves was observed using both light microscopy and SEM. Light micrographs revealed that the growth of neurons differed significantly between when they were on the SRG and on a smooth surface. In Fig. 3 (a), the neurites orientation appears both parallel and perpendicular to groove direction compared to the control in Fig. 3 (b). On the smooth surface without the groove, the neurites expanded in random direction. On the groove-patterned surface, the neurites mostly grow in perpendicular orientation while some neurites extends in parallel orientation with relatively short length. This shows that the contact guidance of neurons has the capability of steering the bi-directional growth of neurites.

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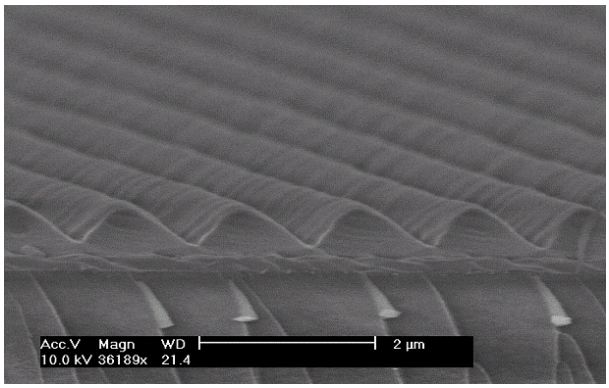
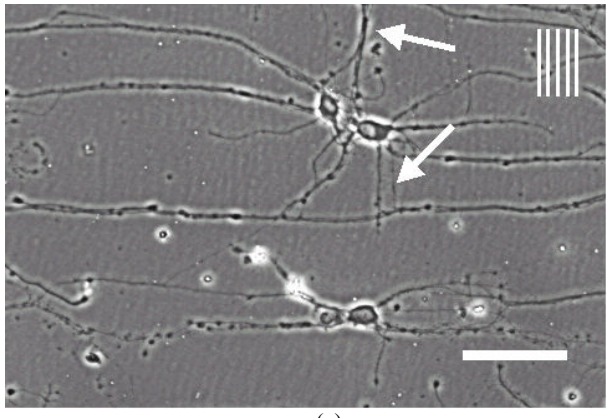


Figure 2: Scanning electron microscope picture of photofabricated surface of azobenzene copolymer. The regular sinusoidal grooved structure had 1.3 µm periodicity and 400 nm modulation depth.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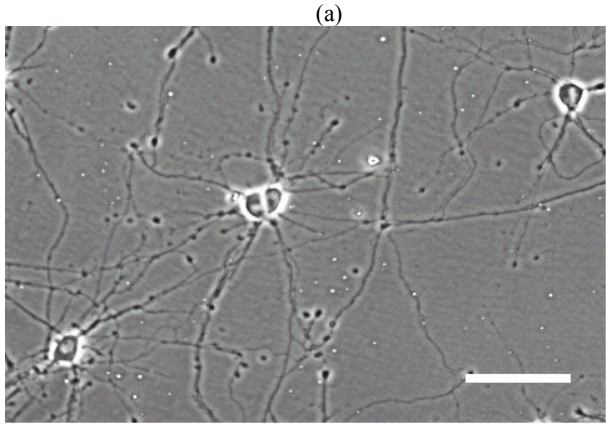
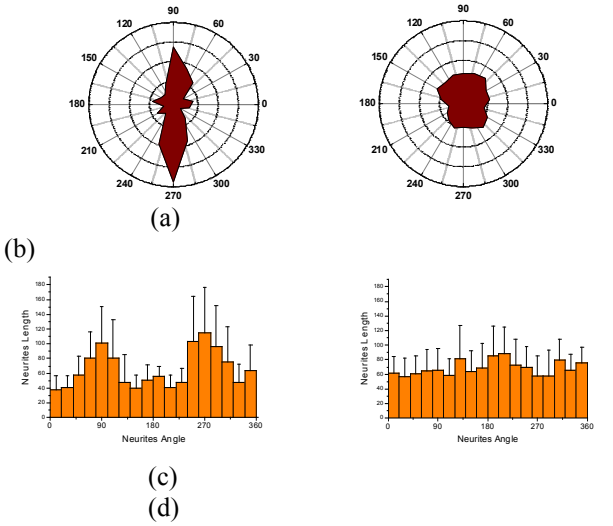

(b)

Figure 3: (a) Hippocampal neurons after 6 days in culture on SRG pattern. The neurites grow perpendicular to groove direction (Narrow lines on upper right corner). White arrows indicate parallel growth of neurites. (b) Hippocampal neurons after 6 days in culture on azobenzene copolymer without SRG pattern. The neurites grow in random direction. Scale bar=50 µm.

Figure 4 presents the statistical data of neurites angle and length on grooved surface and smooth surface. The angle and length of a neurite was measured by drawing a straight line from middle of cell body to middle of growth cone and the parallel direction to microgrooves were defined as 0° degree. Compared to the uniform directions of neurons cultured on smooth surface of the polymer, the neurons are aligned in perpendicular direction to grooves. Though the perpendicular growths were dominant, relatively short lengths of neurites were also observed as described in Fig. 3. In addition to angle of neurites, the lengths of neurites were influenced by the grooved pattern. On the micropatterned surface, the growths of neurites in perpendicular direction were stimulated and measured length was longer than the average of neurites on smooth surface. In parallel direction, the growth was suppressed and shorter than neurites on smooth surface. This suggests that the grooves play a role of the perpendicular contact guidance on the neural cells. The frequencies and of the neurites orientation and growth rate of neurons were significantly influenced by the microgroove structure.



(a)

(b)

(c)

(d)

Figure 4: Statistical data of the growth of neuron on the microtopography. (a) Frequencies of neurites on patterned surface, (b) frequencies of neurites on smooth surface, (c) length of neurites on patterned surface, and (d) length of neurites on smooth surface.

Figure 5 shows the localized topographic effect on the growth of neuronal process. When the neurites of a neuron with its cell body outside of the grooved region grew into the microfabricated region, the neurites adjusted their trajectories to the direction perpendicular to the grooves. This observation further supports that the direction of growth of living neurons can be controlled using photofabrication with laser holography. This is a promising method for controlling the in-situ navigation of living neurons because the fabrication is conducted in an optical, noncontact manner, and no debris remains on the surface of polymer after irradiation.

Figure 6 shows a single neuron cultured on SRG, whose neurite grew out from the cell body perpendicular to the grooves. The repeating microgrooves provided a continuous topographical cue

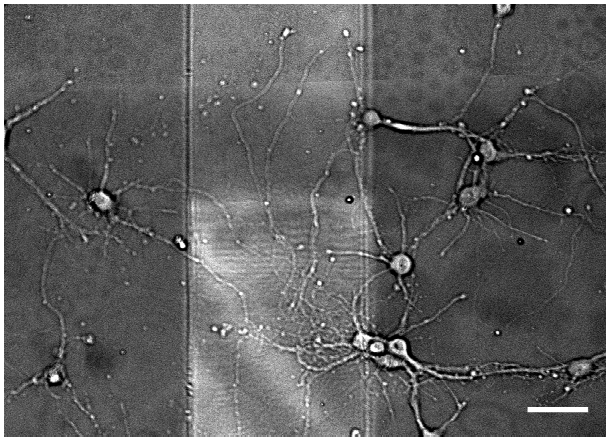


Figure 5: Differential interference contrast micrograph of hippocampal neurons cultured on a photoresponsive polymer with the SRG (1.2 μm wide, 600 nm deep) running horizontally in the middle light region. Neurites extending into the grooved region tended to grow perpendicular to the SRG. Scale bar = 50 μm.

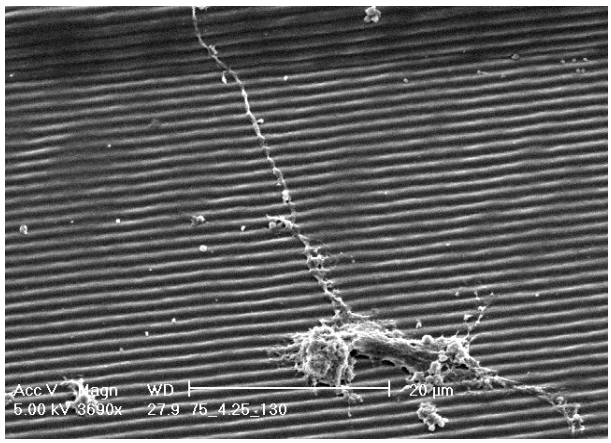
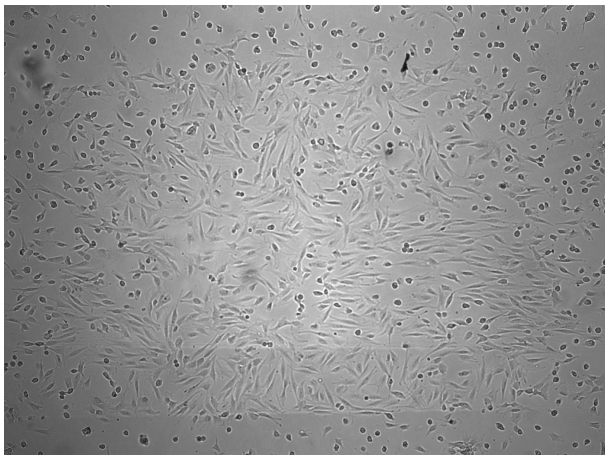


Figure 6: SEM image of rat hippocampal neurons attached on SRG (1.4 μm wide, 400 nm deep), clearly showing a neurite growing out from a cell body perpendicular to the direction of microgrooves. White arrow indicates the site of cell body, where an axon emerged and initiated to extend.

to the neuronal behavior. It has been reported that a grooved substrate determines the site on a hippocampal neuron body from which neurites grow: axons emerge from the perpendicular regions [12]. Therefore, it can be presumed that Fig. 6 shows one long axon growing out from perpendicular regions on the cell body. This implies that a photofabricated structure has a functional influence on the initial differentiation of neurons.

Two Dimensional Patterning of Fibroblast Cells: NIH Fibroblast cells were trypsinized and seeded on the photofabricated polymer surface. Figure 7 presents the fibroblast cell behavior in the early stage of cell spreading after seeding. In Fig. 7 (a), SRGs were inscribed only in the half circle region in the middle. The behavior of cells on the photofabricated region with microgrooves significantly differed from that on untreated region. Figure 7 (b) and (c) are showing the enlarged images of Fig 7 (a): (b) photofabricated region and (c) untreated smooth region. In Fig. 7 (b), cells firmly attached to the substrata and extended their lamellipodia and locally aligned in one direction, while cells in Fig. 7 (c) showed round shapes and poor attachment to substrata.

To evaluate the topographic effect on fibroblast cell attachment and proliferation, we studied the comparison between grooved pattern and simple ablation. Two samples were prepared by irradiating with Ar+ laser in identical duration. In one sample, laser beams were polarized and interfered on the surface of the polymer to generate grooved structure. In the other sample, laser beams were only polarized and employed to the polymer. The durations and intensities were maintained identically except polarization states of the lights. From the AFM analysis (Figure not shown), the scales of the roughness pattern were approximately tens of nanometers, which was considerably negligible compared with SRG pattern. To assess the influences of



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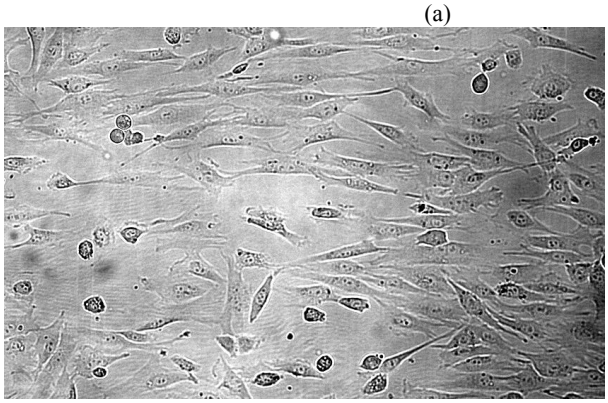

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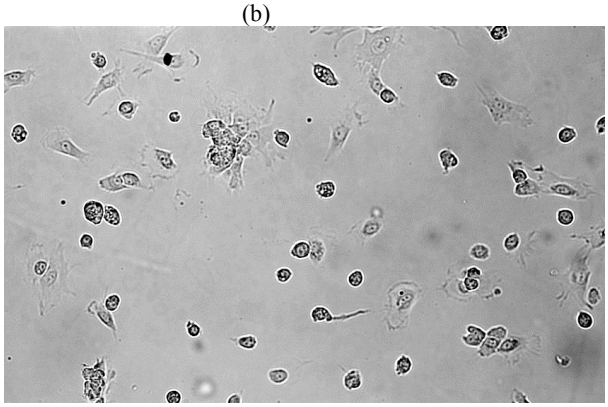


(c)

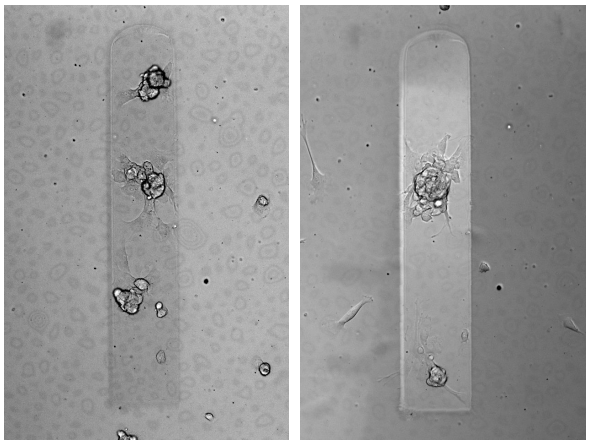
Figure 7: The response of NIH3T3 fibroblast cells on photofabricated polymer surface (a). The cells were seeded (b) on the microgrooved polymer, (c) on the flat surface of polymer. Scale bar = 50 μm.

topographic features on the cell patterning, fibroblast cells were seeded on the polymer with same density and observed in the first day and second day after seeding.

Figure 8 demonstrates the results of cellular behavior on the patterning with simple laser ablation. On the first day, cells were aggregated in small clusters and piled up onto the cells which attached to the polymer. The adhesive contact of fibroblast cells to the laser ablated surface was weak to support the growth of the cells, resulting in failure in proliferating on the pattern on the second day.

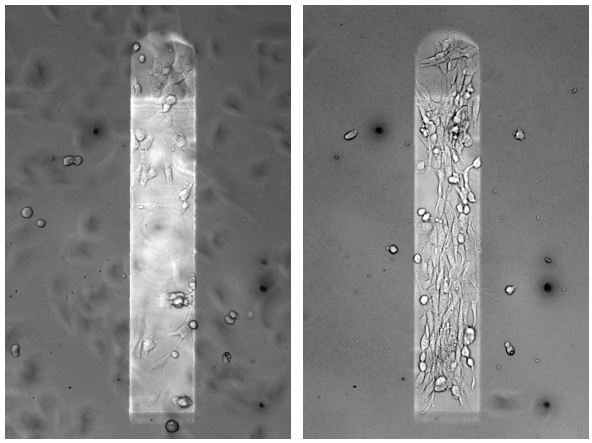
The observation of cellular patterning with grooved topography is presented in Fig. 9. On the first day, the seeded cells were preferably attached to the patterned polymer and grew extending the cell body to the substratum. One day later, the cells were proliferated on the patterned region, confirming the successful spatial patterning of fibroblast cells. This result reveals that the topographic feature of microgroove is effective method to control the attachment and proliferation of fibroblast cells.

Scanning electron microscopy was also used to assess cells attachment (Fig. 10). The cells adhered to the SRG patterned region only. The nuclei of the cells were observed predominantly after fixation. Wrinkles on the polymer were produced during a dehydration process of cell fixation. Due to weak solubility of azopolymer in ethanol, the bottom of the polymer film was separated from the glass substrate. SEM observation revealed that the lamellipodia of fibroblast cells were mostly confined in the laser treated region (Fig. 10 (b)).



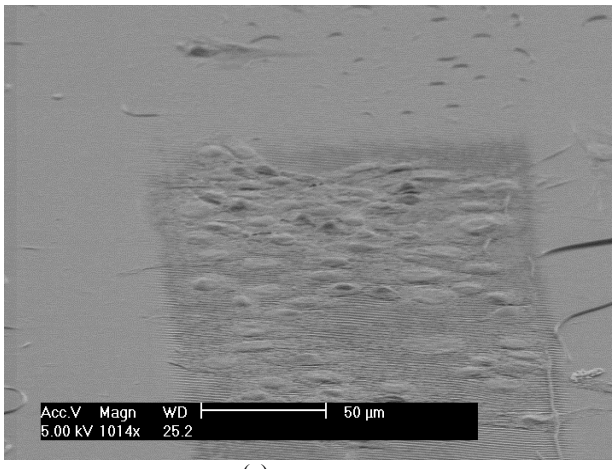
(a) (b)

Figure 8: Fibroblast cells on Laser ablated patterned polymer (a) after cell seeding, (b) 1 day later.



(a) (b)

Figure 9: Fibroblast cells groove-patterned polymer (a) after cell seeding, (b) 1 day later



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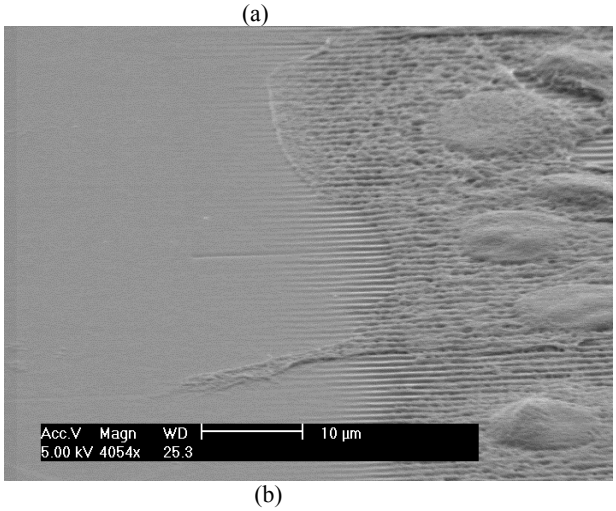

(b)

Figure 10: Scanning electron microscope image of NIH3T3. (a) fibroblast cells attached in a high density on the patterned polymer, (b) the cells on the micropatterned polymer revealed well-developed cytoplasmic extension of lamellipodia.

Conclusions

Laser holographic fabrication has been used to produce micro- and submicroscaled grooves on the surface of azobenzene copolymer. The widths and depths of the grooves were controlled by adjusting the angle and intensity of the irradiating laser beam. On the photofabricated microfeatures, rat hippocampal neurons demonstrated a preference for growing perpendicular to the grooves. The region of irradiation was spatially controlled by a shielding mask to produce localized surface topography, which subsequently induced extending neurites to change their direction of growth. NIH3T3 fibroblast cells were also cultured on the patterned substrate. The cells attached firmly to the patterned region and proliferated in the confined area. Two dimensional cellular patterning can be modulated further process since the photoresponsive polymer is reversible and repeatable. Our experiments indicate that laser holographic fabrication on azobenzene copolymer can direct neuronal growth and influence the initial differentiation of a neuron. This could be used to guide neurons and fibroblast cells in the construction of living cellular networks with the applications of biomedical devices, tissue regeneration, and fundamental studies of cellular processes.

References

[1] Whitesides G., Ostuni E., Takayama S., Jiang X., Ingber D. (2001): 'Soft lithography in biology and biochemistry', *Annu. Rev. Biomed. Eng.*, **3**, pp. 335-73.

[2] Stenger D. and Mckenna T. (1994): 'Enabling technologies for cultured neural networks', (San Diego academic press), p. 4.

[3] Curtis A. and Wilkinson C. (1997), 'Topographical control of cells', *Biomaterials*, **18**, pp. 1573-83.

[4] Li N., Tourovskaia A., Folch A. (2003), 'Biology on a chip: microfabrication for studying the behavior of cultured cells', *Crit. Rev. Biomed. Eng.*, **31**, pp. 423-88.

[5] Patel N., Poo M.(1982): 'Orientation of neurite growth by extracellular electric fields', *J. Neurosci.*, **2**, pp. 483-496.

[6] Ehrlicher A., Betz T., Stuhrmann B., Koch D., Milner V., Raizen M., Käs J. (2002), 'Guiding neuronal growth with light', *Proc. Natl. Acad. Sci. USA*, **99**(25), pp. 16024-8.

[7] Kaji H., Tsukidate K., Matsue T., Nishizawa M. (2004), 'In situ control of cellular growth and migration on substrates using microelectrodes', *J. Am. Chem. Soc.*, **126**, pp. 15026-15027.

[8] Kaehr B., Allen R., Javier D., Currie J., Shear J. (2004), 'Guiding neuronal development with in situ microfabrication', *Proc. Natl. Acad Sci. USA*, **101**, pp. 16104–16108.

[9] Natansohn A. and Rochon P. (2002), 'Photoinduced motions in azo-containing polymers', *Chem. Rev.*, **102** (11), pp. 4139-75.

[10] Chun C., Kim M., Vak D., Kim D. (2003), 'A novel azobenzene-based amorphous molecular material with a spiro linked bifluorene', *J. Mater. Chem.*, **13**, pp. 2904-09.

[11] Jiang X., Li L., Kumar J., Kim D., Tripathy S. (1993), 'Unusual polarization dependent optical erasure of surface relief gratings on azobenzene polymer films', *Appl. Phys. Lett.*, **72**, pp. 2502-04.

[12] Rajnicek M., Britland S., McCaig C. (1997), 'Contact guidance of CNS neurites on grooved quartz: influence of groove dimensions, neuronal age and cell type', *J. Cell Sci.,* **110**, pp. 2905-13.

[13] Walboomers X., Monaghan W., Curtis A., Jansen J. (1999), 'Attachment of fibroblasts on smooth and microgrooved polystyrene', *J. Biomed. Mater. Res.*, **46**, pp. 212-220.

[14] Den Barber E., de Ruijter J., Ginsel L., von Recum A., Jansen J.(1998), 'Orientation of ECM protein deposition, fibroblast cytoskeleton and attachment complex components on silicone microgrooved surfaces'. *J. Biomed. Mater. Res.*, **40**, pp. 291–300.