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Displacement Talbot lithography nanopatterned microsieve array for directional neuronal network formation in brain-on-chip

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Commercial microelectrode arrays (MEAs) for in vitro neuroelectrophysiology studies rely on conventional two dimensional (2D) neuronal cultures that are seeded on the planar surface of such MEAs and thus form a random neuronal network. The cells attaching on these types of surfaces grow in 2D and lose their native morphology, which may also influence their neuroelectrical behavior. Besides, a random neuronal network formed on this planar surface in vitro also lacks comparison to the in vivo state of brain tissue. In order to improve the present MEA platform with the above mentioned concerns, in this paper, the authors introduce a three dimensional platform for neuronal cell culturing, where a linear nanoscaffold is patterned on a microsieve array by displacement Talbot lithography (DTL) and reactive ion etching. Good pattern uniformity is achieved by the DTL method on the topographically prepatterned nonflat surface of the microsieve array. Primary cortical cells cultured on the nanopatterned microsieve array show an organized network due to the contact guidance provided by the nanoscaffold, presenting 47% of the total outgrowths aligning with the nanogrooves in the observed view of field. Hence, the authors state that this nanopatterned microsieve array can be further integrated into microsieve-based microelectrode arrays to realize an advanced Brain-on-Chip model that allows us to investigate the neurophysiology of cultured neuronal networks with specifically organized architectures. © 2016 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/). [http://dx.doi.org/10.1116/1.4961591]

I. INTRODUCTION

Brain on chips assist in studying the circuit-connectivity, physiology, and pathology of neurons and should help us to understand brain functions. The microelectrode array (MEA) is one of the most widely used brain-on-chip platforms to study the neuroelectrophysiology by recording the extracellular potentials of neurons in both in vivo and in vitro. A non-implantable MEA, consisting of an array of multiple microelectrodes, enables long-term simultaneously recordings of signals from a cultured neuronal network. The commonly used commercial MEAs are integrated planar electrodes that measure signals from a neuronal culture, whereby the cells are seeded in a 2D fashion forming a randomly connected neuronal network. This conventional 2D neuronal culture approach has some limitations. For example, neurons attached on the MEA surface in a 2D culture have a different cell morphology compared to their natural state which might influence their behavior in signal transmission, the random neuronal network also has limitations compared to the neural circuits in the brain, since the latter are highly complex networks with particular geometrical organization. Micro- and nanofabrication techniques have been used to engineer three dimensional (3D)-like cell culture systems, to provide micro- or nanotopographical cues to improve neuronal cell adhesion and morphology, as well as to confine the cell network formation into a designed organizational bioarchitecture.

Previously, we have developed a microfabrication process for a silicon microsieving structure consisting of an array of 3D pores including defined, highly uniform aperture sizes with the purpose of parallel positioning of neurons in a 3D microenvironment. Experiments have shown that primary cortical cells cultured in these 3D pores maintain their round cell morphology similarly to the 3D cell culture realized by embedding cells in biogels. Hence, this sieving structure can also be applied, after integration of electrode material, yielding a microsieve electrodes array (μSEA) to establish a neuroelectronic interface by positioning neurons on the...
microelectrodes. Subsequently, the neuroelectronic interface can be utilized for signal recording of neurophysiologic events by culturing a 3D neuronal network atop. We have also previously demonstrated that a surface with nanopatterns, in particular, nanogrooves, can provide topographical cues to guide cell outgrowth in a primary cortical cell network influencing its organization.  

In this paper, we combined the 3D microsieving structure with nanogrooves to develop a nanopatterned microsieve array for achieving a highly guided cell network in culture. Here, displacement Talbot lithography (DTL) was utilized to pattern the planar surface between the pores of the microsieve because of its ability to create submicrometer sized features at high speed (compared to other nanolithography techniques) and thus low cost, as well as its advantage of providing good patterning uniformity atop of nonflat surfaces. Results show that a linear nanoscaffold with a period of 600 nm and a height of 100 nm can be patterned and transferred into the silicon nitride layer of the planar surface of the microsieve with good uniformity by applying DTL and reactive ion etching (RIE). The microsieve array with the integrated nanoscaffold provides contact guidance in the in vitro network formation process of dissociated primary cortical cells. Based on these results, we believe that the nanopatterned microsieve array can help to establish an improved in vitro MEA platform, either a commercial 2D MEA or our novel 3D μSEA, to better study the neuroelectrophysiology in neuronal cell culture experiments in brain-on-chip applications.

II. EXPERIMENT

A. Microsieve array

The design and fabrication of the microsieve array is detailed in our previous work. In brief, the sieves were fabricated by corner lithography and back-etching using (100)-silicon micromachining. The microsieve array contains 900 inverted pyramidal shaped 3D pores with a side length of 20 μm on the top opening [L in Fig. 1(c)], a depth of 18 μm [D in Fig. 1(e)], and an aperture size of 3.2 μm [A in Fig. 1(e)]. These combined pyramidal pores, referred to as a microsieve array, are designed to be used for positioning neurons. The planar surface between the pores of the sieving structure offers a platform for organized network formation among the positioned neurons. Figure 1 shows scanning electron microscopy (SEM) images of the microsieve array, where Figs. 1(a)–1(c) display the 3D pores of the microsieve array, and Figs. 1(d)–1(f) show the microsieve array with integrated polysilicon functioning as contact electrodes, lead wires, and sensing electrodes. The completed μSEA is covered with 250 nm thick silicon-rich nitride (SiRN) serving as the isolation layer deposited by low pressure chemical vapor deposition (LPCVD). The linear nanoscaffold is patterned on this SiRN isolation layer as described in Sec. II B.

B. Nanoscaffold

The grooved nanoscaffold on the planar surface of the microsieve array was fabricated by DTL realized by the PHABLE™ technology (Eulitha). The DTL method utilizes the Talbot effect to realize submicrometer-sized periodic structures (i.e., lines, dots, and circular holes in the photore sist layer) by the self-image of the mask created, in the case of line patterns, resulting in half of the pitch of the original pattern of the mask. Thus, a mask for conventional photolithography can be used to generate features at submicrometer scale with this method. As introduced above, the DTL permits good pattern uniformity on a nonflat surface. However, the topography of the microsieve array may still
cause thickness nonuniformity in the coatings which leads to a poor pattern transfer result. Therefore, we introduced a planarizing layer by precoating a positive resist prior to the DTL experiment to overcome the nonuniformity problem.

Figure 2 shows the fabrication process. The details are as follows: The microsieve array was first cleaned with 99% nitric acid for 5 min to remove the organic contamination followed with cleaning deionized water. After 5 min dehydration bake at 120°C on a hotplate, a layer of primer, hexamethyldisilazane (HMDS) was spin coated at 4000 rpm for 30 s, followed by a positive resist layer Olin OIR 908-35 spin coated at 4000 rpm for 30 s. Due to the topography of the sieving structure, the obtained resist layer on the planar area between the pores is not completely uniform in thickness, as a small amount of resist fills in each pore due to suction of the spin-coating apparatus [Fig. 2(a)]. This resist layer was prebaked on a hotplate at 95°C for 90 s, and then underwent a flood exposure to the UV light (EVC 620 mask aligner) with a reduced dose of 12 mW/cm² for 5 s, so that the exposure dose was efficient to illuminate through the complete resist layer on the planar surface (i.e., between the pores) but not intense enough to illuminate the accumulated resist inside the pores of the sieve [Fig. 2(b)]. Therefore, after a postexposure bake at 120°C for 60 s, followed by 1 min of development in Olin OPD 4262 developer solution (Arch Chemicals), the resist on the planar surface was removed, while a layer of insoluble resist remained inside the 3D pores [Fig. 2(c)]. Subsequently, a layer of bottom antireflective coating (BARC, AZ® BARLi-II 200) was spin coated at 3000 rpm for 45 s, resulting in a thickness of about 180 nm [Fig. 2(d)]., followed with a 160 nm thick positive resist PFI-88 (1:1 diluted with propylene glycol monomethyl ether acetate, Sumitomo Chemical Co.) spin coated at 4000 rpm for 45 s [Fig. 2(e)]. The substrate was then ready for DTL process. A standard mask [Fig. 2(f)] containing a linear pattern with a period of 1200 nm and a linewidth of 600 nm (MHSL400-800, Eulitha) was used for patterning the linear nanoscaffold with a phableR100C DTL system. The exposure setting was optimized according to the approximate equation of the Talbot distance: \(2\pi \gamma/\lambda\), where \(\gamma\) is the pattern period in the mask (1200 nm in this experiment) and \(\lambda\) is the illumination wavelength (375 nm). An optimized DTL range of 12 μm [d in Fig. 2(f)] and a gap of 88 μm [gap in Fig. 2(f)] between the mask and the substrate were used in our DTL experiments. Based on our previous studies on primary cortical cells aligning with different dimensions of nanoscaffold’s period and linear ridge width, we chose a linear pattern with a period of 600 nm and a ridge width of 230 nm. In the mask, the line pattern with 1200 nm period with a 600 nm pitch would result in at most a linewidth of 300 nm in the resist layer (600 nm period and 300 nm ridge width). However, by tuning (i.e., increasing) the exposure dose, the patterns with a narrowed ridge width can be achieved. In fact, the optimal exposure dose for our experiments was 85 mJ (1 mW/cm², exposing 85 s). After developing the resist layer in Olin OPD 4262 developer solution (Arch Chemicals) for 1 min [Fig. 2(g)], the patterns were then transferred into the silicon nitride surface of the microsieve array by reactive ion etching [Fig. 2(h)], with a parallel plate RIE system (Tetske, MESA+ nanolab cleanroom, University of Twente), resulting in features with 100 nm height in the silicon nitride surface. A nitrogen based recipe was used for transferring the pattern of the photoresist 1:1 into the BACR layer, without a loss of resolution. N₂ gas of 50 sccm with 10 mTorr pressure, and plasma with a power of 60 W were used to achieve an etch rate of about 0.3 nm/s. When the BARC layer was fully opened in the space area of the resist ridges, a plasma with 7.5 sccm CHF₃ and 42.5 sccm N₂, 20 mTorr pressure, and 60 W power was used to etch the silicon nitride with an etch rate of 40 nm/min. This microsieve array was then cleaned with 99% nitric acid followed with deionized water to remove the polymer residue (Olin OIR 908-35, AZ® BARLi-II 200, PFI-88) and other organic contamination [Figs. 2(i) and 2(j)].

Before using the microsieve array for cell culturing, it was immersed in 70% ethanol for at least 1 h for sterilization and was then air-dried in a biology safety hood. In order to improve cell adhesion on the nanopatterned surface, the microsieve array was coated with a monolayer of polyethyleneimine (PEI) via immersion in a PEI solution (50 μg/ml in MilliQ water) at 37°C overnight. The PEI coated microsieve array was taken out of the PEI solution and air-dried in the biology safety hood prior to cell seeding [Fig. 2(k)].

### C. Cell culture

Primary cortical cells were isolated from new-born rat’s brain (Mother rat: Wistar Crl:WU) and dissociated in R12H medium, then seeded on the microsieve array in a sterilized petri dish with an approximate amount of 1.2 × 10⁵ cells on the sieve area of the array. Cells were cultured with R12H medium in a controlled atmosphere (5% CO₂, 95% air) at 37°C.
medium containing 100 unit Penicillin and Streptomycin, at 37°C, 5% CO₂, and 95% humidity. The medium was refreshed every 2 days until the culture was terminated.

D. Fluorescent cell staining

Live/dead cell viability assay (Cellstain double staining kit, 04511, Sigma Aldrich) was performed to show the cell network of the living culture on the patterned microsieve array at 11 DIV.

E. Dehydrating cells for scanning electron microscopy

Cells were dehydrated for scanning electron microscope imaging as follows. The cells were first fixed with 4% formaldehyde in 0.1 M phosphate buffered saline (PBS, Sigma Aldrich, D8537) for at least 30 min and were then gently rinsed with PBS for three times. The initially fixed cells were dehydrated by being immersed in 2:1 (v:v) ethanol and HMDS, 1:2 (v:v) ethanol and HMDS, and two times 100% HMDS in sequence, each immersion step for 15 min. Finally, the HMDS was removed and the cells were air-dried for SEM imaging.

F. Microscopy

Atomic force microscopy (AFM; Bruker) was used to characterize the structure of the nanoscaffolds. AFM data were recorded and depicted as 3D models with Nanoscope software (Bruker Corporation). Sectional profiles of the nanoscaffolds were drawn with Origin (OriginLab).

SEM (Sirion High Resolution SEM, FEI) was used to characterize the nanoscaffold on the microsieve array as well as the interface between the cells and the nanoscaffolds with more details.

Optical fluorescence microscopy (Leica, DMI5000M) was used to observe and image the fluorescent stained cells. Images were acquired with the Leica application suite software (Leica Microsystems, LAS05160).

III. RESULTS AND DISCUSSION

A. Uniformity of the nanoscaffold patterning on microsieve array surface

The microsieve array has a thickness of approximately 18 μm, a pore opening length of 20 μm, and an aperture size of 3.2 μm. The pores are evenly distributed in a circular area with a radius of 1.2 mm and a pitch of 70 μm between adjacent pores. These dense and high ridge-to-valley features, i.e., a very topographical structures, can cause wavy patterns which is with unwanted thickness nonuniformity in the spinned coatings, i.e., the BARC and the photoresist layer. The advantage of using the DTL method is that the effective image does not depend on the distance between the mask and the substrate since it is a noncontact exposure method, hence a good pattern uniformity can be ensured on topographical structures. However, for optimal pattern definition, the photoresist layer should be as uniform as possible. Moreover, the subsequent pattern transfer process after the DTL illumination step requires opening the BARC layer. Severe nonuniformity

![Fig. 3](image-url) (Color online) Improved uniformity of the resist coating of PFI-88/BARC on the microsieve array. The resist layer of PFI-88/BARC was more uniform (a) after precoating a planarizing positive resist layer of Olin OIR 908-35 into the pores prior to applying BARC and PFI-88 (b), compared to the coating uniformity of PFI-88/BARC without such a planarizing layer (c). (d)–(f) depict the surface profiles of the planar area between the pores in (a)–(c), respectively.
in coating thickness will cause difficulties in this etching step, and hence leads to relatively poor pattern transfer. The use of a planarizing resist precoating layer helps to reduce the depth difference in the substrate, hence improves the uniformity of the follow-up coatings that are utilized to realize the nanoscaffold. Figure 3 shows the improved uniformity of the PFI-88/BARC coating (a) with a precoating layer of Olin OIR 908-35 in the pores (b) compared to the coating uniformity without such a planarizing precoating layer (c). The uniformity can be judged from the color of the coating with a bright field optical microscope. The deviation in the coating thickness was measured by a surface profiler (Dektak 8, Bruker). Figures 3(d)–3(f) show the profile of the surface topography of the planar area between the pores in (a), (b), (c), respectively. Results show that the deviation in coating thickness was reduced on the sample with a Oli OIR 98-35 precoating layer [Figs. 3(a) and 3(d)], compared to the sample directly coated with PFI-88/BARC [Figs. 3(c) and 3(f)] which had a deviation of around 300 nm in the coating thickness in the 50 μm distance between the pores.

B. Optimization of the exposure setting

As described in Sec. II, when using the pattern with a period of 1200 nm and a ridge width of 600 nm in the mask for the DTL method, the resulted pattern in the resist is with a period of 600 nm and a maximum ridge width of 300 nm. Increasing the exposure dose will result in a narrower ridge width. Table I shows the effect of the increased exposure dose on the feature size. The optimization was carried out on (100)-silicon wafer with a layer of 200 nm thick SiRN deposited by LPCVD. We therefore used 85 mJ for patterning the microsieve array. Good reproducibility was achieved both on the silicon wafers and on the microsieve array samples (N = 4. N: independent samples).

C. Characterization of the nanoscaffold

The nanoscaffold patterned in the planar SiRN surface between the 3D pores using the optimized DTL settings was characterized with SEM imaging and AFM scanning (Fig. 4). The pattern shows good uniformity on the planar SiRN area [Fig. 4(a)] Although a precoating step indeed highly improves the thickness uniformity of the PFI-88/BARC coating (Fig. 3), a pattern-free area around the edge of the sieve remained, because the thinner polymer layer near the pore opening was consumed during the BARC removal etch process. Nevertheless, the pattern that filled the interspace surface is efficient enough for providing topographical guiding cues for the neuronal cell outgrowth, since the length of these outgrowths are at least of tens of microns in average while the pattern-free area is no larger than 5 μm.

D. Cell alignment on nanoscaffold

Viability staining of the cells helps to present the cell network formed on the microsieve array. Figure 5 shows the living cell staining results. The fluorescent square areas in Fig. 5(a) imply that a cell was located in or partly into one of the pore structures. Figure 5(b) provides a closer view of the cell.
network. Aligned outgrowths that extend parallel to the direction of the nanogrooves can be observed in this picture. About 47% of the total amount of the outgrowths aligned with the nanogroove’s direction in the observed area. Figure 6 further confirms the alignment of the cell outgrowths with the nanogrooves and shows that a cell that is positioned in the pore structure is able to extend its outgrowths following the guidance of the nanogrooves. More details of the analysis of the aligned outgrowths on the nanoscaffold is provided in the supplementary material.\textsuperscript{20}

As introduced earlier in this paper, this sieving structure can be further applied to establish a neuroelectronic interface by positioning neurons when microelectrodes are integrated on this platform, which is not yet the core of our research project at this stage. As a proof-of-principle, the guided primary cortical cell network on the nanopatterned microsieve array shows that by introducing a nanogrooved scaffold onto the planar surface area between the pores, the microsieve array platform is capable of providing guidance to the positioned neuronal cells to form an organized neuronal network, which may have profound influence on the neuroelectric activities of the cells, as well as the data configurations of the recorded signals.

IV. CONCLUSIONS

In this paper, we introduced a linear patterned nanoscaffold for guiding neuronal cell network formation on a microsieve array utilizing displacement Talbot lithography. With the DTL method, submicron linear patterns were successfully fabricated on the prepatterned topographical surface of a microsieve array after applying a planarizing positive photoresist layer. The nanoscaffold in the silicon nitride surface, with a period of 600 nm, a ridge width of 230 nm, and a height of 100 nm, provides effective contact guidance for a directional neuronal cell network formation. This nanopatterned microsieve array can be further integrated into an advanced microelectrode array platform to investigate the effect of the network organization to the neuroelectrophysiology of the neuronal cells.

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Fig. 5. (Color online) Directional cortical cell network revealed by living cell staining. (a) A large view of the cell network on the microsieve array. The fluorescent squares indicate a living cortical cell grow in or partly into the pore. (b) A zoomed-in view of the cell network. The dashed squares indicate three pores on the microsieve array. The arrow indicates the direction of the nanogrooves.

Fig. 6. Scanning electron microscope images of the cells on the microsieve array. (a) The outgrowths extending from a cell (a glial cell) aligned with the nanogrooves. The asterisk indicates the cell soma, and the arrows indicate the outgrowths. (b) A detailed image of the outgrowth alignment.
dissociated cells (Biomedical Signals and Systems, MIRA Institute). The authors thank A. J. Bastiaens (Microsystems Group and ICMS Institute for Complex Molecular Systems, Eindhoven University of Technology) for his help with analyzing the alignment result of the cells.

20See supplementary material at http://dx.doi.org/10.1116/1.4961591 for details of the analysis of the aligned outgrowths on the nanoscaffold with Fast Frontier Transform algorithm.