Investigating the interplay of lateral and height dimensions influencing neuronal processes on nanogrooves

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Investigating the interplay of lateral and height dimensions influencing neuronal processes on nanogrooves

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In this work, nanogroove dimensions as a design input parameter for neuronal differentiation and neurite outgrowth in brain-on-a-chip (BOC) applications are investigated. Soft lithography in polydimethylsiloxane (PDMS) is used extensively in organ-on-a-chip applications to create environments for in vitro models. As such, it is used to fabricate cell culture substrates with nanogrooved patterns. Using a newly developed analysis method, the effect of the nanogrooved, biomimetic PDMS substrates is compared with lateral and height variations within the nanometer range as measured by means of atomic force microscopy (AFM). PDMS culture substrates were replicated from a cyclic olefin copolymer template, which was fabricated either directly by thermal nanoimprinting from a jet and flash imprint lithography (J-FIL) resist pattern (process I) on a polished silicon wafer or via an intermediate reactive ion etched all-silicon mold (process II) that was fabricated by using the J-FIL resist pattern as in process I as a mask. To study the interplay between the lateral and height dimensions of nanogrooves on the differentiation process of SH-SY5Y cells, which are a well-established model for neuronal cells that form networks in culture, the authors first characterized the feature sizes of the PDMS substrates received from both processes by AFM. On average, nanogrooved patterns from process I had a $1.8 \pm 1.1\%$ decrease in pattern period, a $15.5 \pm 12.2\%$ increase in ridge width compared to the designed dimensions, and a height of $95.3 \pm 10.6\ nm$. Nanogrooved patterns for process II had a $1.7 \pm 1.7\%$ decrease in pattern period, a $43.1 \pm 33.2\%$ increase in ridge width, and a height of $118.8 \pm 13.6\ nm$. Subsequently, they demonstrated that neurite outgrowth alignment was particularly strong if the pattern period was 600 nm or 1000 nm with the additional constraint for these patterns that the ridge width is $<0.4$ times the pattern period. Increasing pattern height increased the fraction of differentiated cells within the cell culture and increased neurite length, but had no direct impact on outgrowth alignment. This study forms the basis for optimization in the bottom-up engineering of neuronal network architecture, for which specific patterns can be selected to assist in neuronal cell differentiation and direct neurite growth and alignment. Such organized neuronal networks can aid in the design of in vitro assay systems for BOC applications by improving biological response readouts and providing a better understanding of the relationship between form and function of a neuronal network. © 2018 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/).

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I. INTRODUCTION

Advances in microphysiological systems to study cell physiology and cell pathology in vitro, known as organ-on-a-chip, rely on micro- and nanoscale fabrication methods to produce biocompatible microenvironments that emulate the microarchitecture of tissues. In this context, micro- and nanogrooves induce physical cues that stimulate directional cell morphology and aligned neuronal networks. This is the case with brain cells where nanogrooves guide the direction of neuronal outgrowth (neurite). Hence, the dimensions of such topographical patterns can be used as a design feature in brain-on-a-chip (BOC) applications. Various lithographical techniques achieve features that provide nanotopographical cues and hence mechanotransduction to neuronal cells in culture, influencing the phenotype of the cells. More specifically, in vitro experiments demonstrate that nanotopography influences neurite length and orientation and neuronal polarity and promotes stem cell differentiation toward the neuronal lineage. Here, we focus on the fabrication of nanogroove cell culture substrates and the analysis of the cellular response in a neuronal network. The fabrication of larger structures such as microgrooves is also feasible and has shown that neurite guidance is possible at the microscale. While microgrooves allow contact guidance, experiments on microgrooves have shown that neuronal differentiation is not significantly enhanced. Topographical cues of nanogrooves may interact with either chemical or mechanical cues, as shown by current research with a large variety of cell types and nanogroove dimensions.
Previously, research demonstrated that the nanogroove dimensions must be optimized for a specific cell type. As such, experimental trial-and-error is necessary to gain insight into the effect of topographical cues on neurite and neuronal network properties. It is therefore important to use effective ways to investigate the optimal use of nanogrooves in the bottom-up engineering of neuronal cell cultures for BOC applications.

Our approach to facilitate the selection of preferable nanogroove dimensions uses an image-based screening method. The nanogrooves were fabricated by jet and flash imprint (J-FIL) lithography, as an arrangement of 27 patterns with lateral dimensions in the range of 200–2000 nm. Previous experiments have shown that nanogrooves with lateral dimensions in the order of 400–600 nm and a height of approximately 118 nm induce a strong alignment of neuronal outgrowths of primary rat cortical cells in culture.5 Our recent results also include height variations that confirm this alignment capability on similar patterns for the commercially available, well-established neuroblastoma cell line, SH-SY5Y. Here, neurite outgrowth alignment is particularly strong if the pattern period is within 400–600 nm or at 1000 nm with the additional constraint of ridge widths of <0.4 times the pattern periodicity. The effect of the lateral dimensions has been investigated by both ourselves8,23 and several other authors.5,7,9,11,12 To some extent, vertical dimensions were also investigated.5,11 However, the height of nanogrooved patterns and the effect of slight variations in nanogroove height in the range of 80–140 nm should be investigated in more detail.

Here, it is our aim to investigate nanogroove dimensions as a design input parameter for neuronal differentiation and neurite outgrowth in BOC applications.8,23 For this purpose, we compared the morphology and differentiation of SH-SY5Y cells on nanogrooves with height variations. Previously, our group has used soft lithography to fabricate cell culture substrates with nanogroove patterns in polydimethylsiloxane (PDMS). To ensure that only variations in nanogroove dimensions would play a role in the cell culture experiments, a replica molding process was used to obtain nanogrooved PDMS cell culture substrates from the two different molds. In this work, we achieve this by replicating the PDMS substrates from a cyclic olefin copolymer (COC) template either directly fabricated by thermal nanoimprinting from a J-FIL resist pattern on a polished silicon wafer or via an intermediate reactive ion etched (RIE) all-silicon mold, which was fabricated by using the same J-FIL resist pattern as a mask.

The results of the SH-SY5Y cell culture experiments were analyzed through image-based screening of cell morphology and neurite properties for all made patterns. Statistical analysis was performed to identify significant differences between patterns. Our study allowed us to investigate nanogroove height variations as a design input parameter in influencing neurite outgrowth for BOC applications. Such organized neuronal networks can aid in the design of in vitro assay systems for BOC applications, for example, by improving the biological response readouts and by providing a better understanding of the relationship between form and function within neuronal networks.

II. EXPERIMENT

A. Fabrication of nanogrooved molds for replica molding

Details of fabrication of nanoresist as scaffolds were published previously by Xie and Luttge.8 In brief, nanoresist was patterned by J-FIL on a standard double-sided polished 100 mm diameter silicon wafer coated with a bottom antireflective coating (DUV30J, Brewer Science) layer using a quartz master kindly provided by the Bijkerk group at the University of Twente. Nanogrooved pattern periodicity ranged from 200 to 2000 nm with a ridge width of 100–1340 nm resulting in 27 different patterns with a height of 118 nm. Subsequently, nanoresist patterns (i) were either used directly as a template in thermal nanoimprint lithography or as a mask in pattern transfer by means of RIE, yielding a silicon template (ii) [Fig. 1(a)]. RIE was performed using CHF3/argon gas composition with an etch rate of approximately 0.8 nm s−1 for the polymer layer and an SF6/C4F8 gas composition with an etching rate of approximately 10 nm s−1 for silicon.24

Subsequently, a negative copy in COC (optical grade TOPAS 8007S-04, Kunststoff-Zentrum) of each of the molds (i) and (ii) was made by thermal nanoimprinting at 108 °C and by applying a pressure of 4 MPa using a thermal nanoimprint lithography system (EITRE 6, Obducat) [Fig. 1(b)]. COC was kept on molds i and ii, respectively, until cooled to room temperature prior to peeling off the COC, resulting in a secondary mold made from COC and labeled iii and iv, respectively.

B. Nanogrooved cell culture substrates

The nanogrooved cell culture substrates were made by PDMS replica molding using Sylgard® 184 (Dow Corning, Germany) for the two routes of fabrication, processes I and II [Fig. 1(c)]. PDMS elastomer and curing agent were mixed at a weight ratio of 10:1 and degassed for 10 min using a vacuum chamber prior to spin-coating a 100 μm layer of PDMS onto the COC molds and placing the PDMS-covered molds into an oven at 65 °C for 4 h to fully cure the PDMS. PDMS was peeled off COC molds iii and iv, resulting in PDMS substrates v and vi, respectively. The PDMS cell culture substrates were subsequently placed in a polystyrene Petri dish prior to further use.

The nanogrooved patterns on the PDMS substrates were surrounded by an area of flat PDMS. The flat PDMS was used as a control surface to compare the effect of the nano-grooved patterns against a nonpatterned surface for neuronal cell morphology.

C. Characterization of nanogroove dimensions

Tapping mode atomic force microscopy (AFM; XE-100, Park Systems) was used to characterize the dimensions of the
nanogrooved patterns in PDMS substrates $v$ and $vi$, using noncontact cantilevers (PPP-NCHR, Park Systems). AFM data were recorded with XEP software (Park Systems), and data were analyzed using GWYDDION software.\textsuperscript{25} Nanogrooved pattern dimensions were calculated for PDMS substrates from processes I and II, as listed in Table I.

D. Neuronal cell culture on nanogrooved PDMS

The nanogrooved PDMS substrates from processes I and II were sterilized for 5 min using 70% ethanol, and the substrates were placed in an oven at 65 °C for 1 h to ensure that ethanol had fully evaporated. A coating of $10 \mu g$ cm$^{-1}$ fibronectin (FC010, Sigma Aldrich) in phosphate buffered saline (PBS; LO BE02-017F, Westburg) was applied for 30 min to the PDMS nanogrooves prior to seeding cells in a Petri dish. After coating the surface with fibronectin, Petri dishes with PDMS substrates were used for cell culture by aspirating the fibronectin solution from the dish and immediately adding cell suspension onto the surface.

To study neuronal differentiation and network guidance based on nanogroove dimensions, including height variations, the human neuroblastoma SH-SY5Y cell line (# 94030304, Sigma Aldrich) was cultured on the PDMS substrates received from processes I and II. In brief, cells were removed from cryovials stored in liquid nitrogen and thawed, plated in a T75 flask, and cultured to 70%–80% confluency prior to use in the experiments according to the manufacturer’s protocol. Cells were cultured in Dulbecco’s modified Eagle’s medium and Ham’s F12 medium in a 1:1 ratio (L0093-500, VWR) supplemented with 10% fetal bovine serum (SFBS lot 11113, Bovogen) and 1% penicillin/streptomycin (LO DE17-602E, Westburg) in an incubator at 37 °C and 5% CO$_2$. Cells were then seeded at 0 days in vitro (DIV) onto the fibronectin-coated substrates in standard culture medium at 1500 cells cm$^{-2}$. Cells were allowed to adhere to the PDMS cell culture substrates, and the medium was replaced with culture medium supplemented with 10 $\mu$M retinoic acid (R2625, Sigma Aldrich) for 72 h to initiate differentiation of the cells into the neuronal lineage.\textsuperscript{26} The medium supplemented with retinoic acid was replaced after 36 h with new medium supplemented with retinoic acid. Subsequently, at 3 DIV, the medium was replaced with culture medium supplemented with 50 ng ml$^{-1}$ brain-derived neurotrophic factor (B2795, Sigma Aldrich) for 24 h to enhance cell differentiation.\textsuperscript{27} Cells were kept in a standard culture medium until 21 DIV, with the medium being refreshed every 48 h. Cultures were fixed on 21 DIV by washing the samples twice in PBS and subsequently treating them with 3.7% formaldehyde (1.040.031.000, Merck Millipore) for 30 min.

E. Immunofluorescent cell staining

Immunofluorescence staining was performed using anti-β-tubulin III (T8578, Sigma Aldrich) and antimouse IgG Alexa Fluor 555 (A21424, Thermo Fisher Scientific). These proteins were used as primary and secondary antibodies to selectively stain SH-SY5Y cells that had differentiated into a neuronlike phenotype.\textsuperscript{28} Cells were permeabilized for 10 min with 0.1% Triton X-100 (1.086.031.000, Merck Millipore) and incubated for 15 min in a blocking buffer of 10% horse serum (HS; 16050-122, Thermo Fisher Scientific) in PBS, incubated for 1 h with 1:200 primary antibody and 1% HS in PBS and incubated for 1 h with 1:200 secondary antibody in PBS. Additionally, the cytoskeletal protein F-actin was stained using 2 drops ml$^{-1}$ ActinGreen$^{50}$ (R37110, Thermo Fisher Scientific) for 30 min, and cell nuclei were

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**Table I. Parameters derived from AFM measurements on nanogrooves.**

<table>
<thead>
<tr>
<th>Nanogroove pattern feature</th>
<th>Measurement$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>Height difference ridge and groove of pattern (nm)</td>
</tr>
<tr>
<td>Pattern period (D)</td>
<td>Distance between ridges (nm)</td>
</tr>
<tr>
<td>Ridge width (L)</td>
<td>Full width at half height (nm)</td>
</tr>
<tr>
<td>Groove width (G)</td>
<td>D−L</td>
</tr>
</tbody>
</table>

$^a$ $n$ = 5 measurements for each pattern feature.
counterstained with 2 drops ml⁻¹ NucBlue® (R37606, Thermo Fisher Scientific) in PBS for 5 min. Samples were rinsed three times for 5 min with PBS prior to each step. The immunofluorescence staining protocol is based on the standard protocols provided by the supplier.

F. Image analysis

After staining, the SH-SY5Y cells were visualized using immunofluorescence microscopy (EVOS FL, Thermo Fisher Scientific). For each of the patterns, one image was taken of the cell nuclei, F-actin, and β-tubulin III staining with a 10× objective. To determine the degree of neurite alignment in the fluorescence images, a bright field reference image at the edge of each of the patterns was taken using the 10× objective to define the direction of the nanogrooves.

While whole image fast Fourier transform has been used previously to receive information on the level of alignment of cells to a pattern, we applied automated detection of cell bodies and neurites to the images for the analysis of the potential interplay of lateral and height dimensions of the nanogrooves on neuronal processes. This was done by using the commercially available software package, HCA-Vision® (Refs. 29 and 30; version 2.1.5, CSIRO), as a tool to determine the total number of cells, the number of cells with neurites, the neurite length, and the number of neurites and branches per cell in an image.

To evaluate whether the specific features of the nanogrooved patterns had an effect on the orientation of neurites, a Frangi Vesselness filter in MATLAB (R2015b including Image Processing Toolbox, Mathworks) was used to calculate the alignment of each pixel belonging to the identified neurites from HCA-Vision®. Neurites were considered aligned to the underlying pattern of the substrate when the angle of detected pixels was aligned within ±30° of the direction of the pattern as measured from the reference images for each of the nanogrooved patterns.

G. Statistical analysis

Independent cell culture experiments were performed five times for process I and three times for process II. Cell culture data were tested for normality using the Lilliefors normality test, a correction on the Kolmogorov–Smirnov normality test, which does not require a known population mean or standard deviation. Based on the results for the normality test, data were considered not to be normally distributed. Statistically significant differences between cell culture results for the different nanogrooved patterns were tested by means of the nonparametric Kruskal–Wallis test and were considered statistically significant when $P < 0.05$. Data for the cell culture experiments are represented as the median with the interquartile range (IQR) unless stated otherwise. The statistical analysis was performed using MATLAB (R2015b including Statistics and Machine Learning Toolbox, Mathworks).

III. RESULTS AND DISCUSSION

Cell biology data should incorporate a thorough statistical experimental design. Considering the number of cell culture repeats needed to set up such a design comparison study, it is important that the quality of nanogrooves per culture substrate used in the experiment is highly reproducible and a suitable nanofabrication method, i.e., soft lithography, is selected for this purpose.

To demonstrate pattern fidelity in the two routes of pattern transfer for the nanogrooved PDMS culture

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**Fig. 2.** Overview of AFM measurement results for nanogrooved patterns from processes I and II. (a) Representative AFM area scan of a pattern in PDMS from process I, with a period of 800 nm and a ridge width of 230 nm. (b) Representative AFM area scan of a pattern in PDMS from process II, with a period of 800 nm and a ridge width of 230 nm. (c) Line profiles for patterns shown in (a) and (b). (d) Scatterplot showing the groove width versus pattern height for both processes. For patterns with a groove width of less than 200 nm, the pattern height becomes linearly dependent on the cantilever probe tip of the AFM, resulting in incorrect measurements of pattern height. Labels in the legend indicate height measurements for all investigated patterns, referring to fully measured patterns by process 1 and process 2, while process 1* and process 2* refer to patterns that showed linear dependency on the AFM tip.
substrates, the geometrical dimensions were characterized using AFM. Subsequently, a differentiation and alignment analysis of the neuronal cell culture on nanogrooves was performed to identify a potential correlation among the different dimensional parameters. Secs. III A–III D present the results divided by aspects of the fabrication, the analysis of individual cellular processes, and the organization in the neuronal network architecture.

A. Characterization of nanogrooved PDMS culture substrates

Nanogrooves of all patterns were measured for both fabrication routes, processes I and II. Areas of at least \(5 \times 5 \mu m\) in size were measured for process I [Fig. 2(a)] and process II [Fig. 2(b)]. Line profiles were taken from each pattern [Fig. 2(c)] for PDMS substrates, and the actual pattern period \((D)\), ridge width \((L)\), groove width \((G)\), and height \((H)\) were determined for each nanogrooved pattern as shown in Table I. A complete table of these values for all patterns can be found in the supplementary material (Table SI). Patterns are referred to by their pattern period and ridge width. For instance, D800L230 is the name of the pattern shown in Figs. 2(a) and 2(b), for which \(D = 800\) and \(L = 230\) nm in the original design.

1. Limitations of AFM measurements on nanogrooved patterns

The profile height for the patterns was expected to be approximately 100 nm; however, a range of patterns did not return measurements close to this value. Considering the size of an AFM noncontact tapping mode cantilever probe, this is caused by the probe not being able to reach the bottom of the groove. The average tip size of a probe is less than 10 nm, but this tip is 10–15 \(\mu m\) in height and subsequently also broadens from the very tip of the probe to the cantilever base. Hence, the measured pattern height was set out against the measured groove width for all nanogrooved patterns first [Fig. 2(d)] to determine the range of patterns that were still being measured correctly by means of the AFM probe. Patterns that were incorrectly measured showed a linear relationship between groove width and pattern height, reinforcing the statement that the geometry of the probe was the limiting factor when measuring these patterns. For these patterns, no further assessments were made with regard to pattern quality and we only used these patterns in a qualitative, observatory mode of analysis rather than drawing detailed conclusions on how these fine lateral dimensions of the original design influenced the neuronal cell culture. On patterns with a measured height of greater than 75 nm or groove width of at least 200 nm, a quantitative analysis was performed according to Sec. III A 2.

2. Dimensions of nanogrooved patterns

On average, nanogrooved patterns from process I showed a 1.8 ± 1.1% decrease in pattern period, a 15.5 ± 12.2% increase in ridge width compared to the original values of the designed dimensions, and a height of 95.3 ± 10.6 nm. Nanogrooved patterns for process II that were considered had a 1.7 ± 1.7% decrease in pattern period, a 43.1 ± 33.2% increase in ridge width, and a height of 118.8 ± 13.6 nm. For these patterns, all heights for process II were greater than for process I.

In conclusion, for both fabrication processes, the pattern period and pattern height did not change substantially compared to the designed dimensions. However, the ridge width values were greater and had large standard deviations compared to the designed dimensions. These findings can be explained by considering the shape of most nanogrooved patterns as seen in the line profiles. Qualitatively, the pattern dimensions in PDMS were reproducibly retained [Fig. 2(c)]. However, the ridge edges in all cases, in particular for smaller patterns, are rounded and there is a slight slope that connects the groove bottom and ridge top of the patterns. The ridge width is calculated from the full width at half height. Due to the rounded ridges, this method seemed most

![Fig. 3. Examples of differentiated SH-SY5Y cells cultured on nanogrooved PDMS with a theoretical pattern period of 1000 nm and a ridge width of 500 nm. (a) Immunofluorescence staining of cells on a nanogrooved PDMS substrate derived from process I with a height of 103.0 nm. (b) Immunofluorescence staining of cells on a nanogrooved PDMS substrate derived from process II with a measured height of 134.4 nm. Nanogrooves are directed along the horizontal axis of the image as shown by the dashed white double-headed arrows. Cells were stained for the presence of neuron-specific marker β-tubulin III (red), cytoskeletal protein F-actin (green) and stained for cell nuclei (blue). The scale bar denotes 400 μm.](image-url)
Fig. 4. Tukey box plots for neuronal differentiation and neurite alignment parameters comparing patterns from fabrication processes I and II by pattern name. (a) Fraction of differentiated cells in the total cell population for differentiated SH-SY5Y neuroblastoma cells on nanogrooved patterns. (b) Mean neurite length as calculated for each analyzed image of differentiated cells on nanogrooved patterns. (c) Fraction of differentiated cells with branching neurites in the population of differentiated cells. (d) Percentage of aligned neurites as calculated from each analyzed image of the differentiated cells. Patterns denoted by designed dimensions for pattern period, $D$, in nm and ridge width, $L$, in nm. For example, the pattern with $D=800$ and $L=230$ nm is denoted as $D800L230$. Statistically significant differences between patterns are denoted by * for $P < 0.05$. The legend in plot (b) shows which box plots and statistically significant differences refer to either process I or process II, a convention that applies to all plots in this figure. The asterisks located at the pattern name refer to differences between processes I and II for that specific pattern name.

Fig. 5. Comparing neuronal differentiation and neurite alignment for patterns with similar lateral dimensions but different heights. (a) Fraction of differentiated cells in the total cell population for differentiated SH-SY5Y neuroblastoma cells on nanogrooved patterns. (b) Mean neurite length as calculated for each analyzed image of differentiated cells on nanogrooved patterns. (c) Fraction of differentiated cells with branching neurites in the population of differentiated cells. (d) Percentage of aligned neurites as calculated from each analyzed image of the differentiated cells. Statistical significance is shown by ** for $P < 0.01$. 
appropriate but may have enlarged ridge width. Another reason for the enlarged ridge width is the use of RIE in process II, as this process etches away the nanoresist patterns and further etches the patterns into the silicon. During this process, detectable deviations from the original dimensions of the patterns occurred. This is particularly the case for the groove of the pattern, as this would retain a more rounded shape due to etching and is a major reason for choosing to incorporate the COC intermediate molds as direct copying of the etched nanogrooves into PDMS would mean these rounded features end up on the ridges.

B. Analysis of individual cellular processes on nanogrooved PDMS

1. Neuronal cell culture on nanogrooved PDMS

Neuronal cell culture was performed on the PDMS nanogrooved substrates as derived from both processes I and II. An example of the cell culture results is shown in Figs. 3(a) and 3(b) for pattern D1000L500. Cells were kept alive for 21 DIV. Neuronal differentiation was confirmed by staining β-tubulin III. On most patterns, qualitative observations already showed a degree of alignment, as demonstrated by the oval-shaped cell bodies with the major axis in the direction of the nanogrooves and the observation of neurites being grown more preferably in the direction of the grooves.

2. Comparing neurite properties between processes I and II

The influence of a particular pattern on the individual cellular processes for processes I and II was analyzed with regard to overall differentiation, neurite length, branching, and neurite alignment as listed by name (Fig. 4). We postulate that a significant change in these values can be attributed to changes in the actual pattern dimensions for the same given name. The fraction of differentiated cells in the cell population is increased for patterns of process II when compared with those from process I [Fig. 4(a)]. Pattern D450L180 showed the highest differentiation with a statistically significant difference compared to pattern D2000L600 with the lowest differentiation within process II. Patterns D600L130, D100L500, and D2000L1340 showed a statistically significant increase in differentiation for process II compared to process I.

A slight increase was observed for the mean neurite length calculated for each analyzed image [Fig. 4(b)] and for the fraction of cells with branching neurites from the
population of differentiated cells [Fig. 4(c)]. However, neurite length does not increase significantly for any pattern except D2000L1340.

For branching, process II yielded a statistically significant increase for D800L230 and D2000L1340 and between the two processes for patterns D600L130, D1000L500, and D2000L1340. Neurite alignment did not show an increasing trend when comparing processes I and II [Fig. 4(d)]. For process I, a statistically significant difference was observed between D500L130 and D2000L1340 and between D600L180 and D2000L1340. Comparing between processes for alignment showed a statistically significant difference within patterns D600L180, D800L230, and D800L400.

Overall, this information shows that nanogrooved patterns from different fabrication processes have different degrees of influence on neuronal cells. For a more detailed study, it is important to account for the specific dimensions per pattern for the influence of dimensional variation on the neuronal processes, as presented in Sec. III C.

**C. Influence of nanogroove height on the neuronal network architecture at similar lateral dimensions**

From a fabrication perspective and that of utilizing specific nanogrooved patterns within BOC applications, it is important to define detailed differences between differentiation and alignment dependent on the feature dimensions. Hence, one can optimize for certain neuronal behaviors in culture by selecting from a range of nanogroove dimensions. To this end, it is important to compare these patterns that have similar lateral dimensions (within ∼20% difference between the patterns) as measured in the PDMS nanogrooved substrates but different heights.

We hypothesized that cell membrane proteins sense an increase in nanogroove height and are therefore more sensitive to nanotopographical cues during differentiation. Patterns D450L180 and D500L130 were selected as patterns with small lateral dimensions, and patterns D750L180 and D800L230 were selected as patterns with large lateral dimensions for this comparison.

**1. Height variations at small lateral dimensions**

For small lateral dimensions (Fig. 5), the height change between processes I and II was 25.4 nm. The fraction of differentiated cells within the total population did not increase significantly, with a median ± IQR of 0.39 ± 0.15 for process I and 0.48 ± 0.14 for process II. Mean neurite length per image showed a statistically significant increase at $P < 0.01$ of 46.6 ± 8.8 nm for process I and 57.8 ± 6.8 nm for process II. The fraction of cells with branching neurites from all differentiated cells did not significantly increase from 0.01 ± 0.02 for process I to 0.03 ± 0.03 for process II. Additionally, neurite alignment showed a slight decrease from 84.3 ± 8.4% for process I to 81.8 ± 7.1% for process II.

**2. Height variations at large lateral dimensions**

For larger lateral dimensions (Fig. 6), the height change between processes I and II was 23.8 nm. The fraction of differentiated cells within the total population showed a statistically significant increase at $P < 0.05$, with 0.30 ± 0.13 for process I and 0.36 ± 0.03 for process II. Mean neurite length per image did not increase significantly from 42.7 ± 10.7 nm for process I to 51.1 ± 20.4 nm for process II. The fraction of cells with branching neurites from all differentiated cells did not significantly increase from 0.02 ± 0.04 for process I to 0.01 ± 0.04 for process II. Neurite alignment showed an increase from 77.7 ± 13.4% for process I to 81.4 ± 11.12% for process II.

**D. Influence of height and ridge to period ratio on the neuronal network architecture**

From the results of the large and small patterns with similar lateral dimensions, several observations were made. First, differentiation and neurite length were increased by the increase in height for both small and large patterns. Second, the IQR for differentiation decreased as height increased. This showed that a ∼25 nm increase in height helped SH-SY5Y cells to differentiate and created longer neurites but did not necessarily aid alignment.
Fig. 9. Comparing neuronal differentiation and neurite alignment for patterns with a similar ridge width (L) relative to pattern period (D) where L/D = 0.38 and heights were similar. (a) Fraction of differentiated cells in the total cell population for differentiated SH-SYSY neuroblastoma cells on nanogrooved patterns. (b) Mean neurite length as calculated for each analyzed image of differentiated cells on nanogrooved patterns. (c) Fraction of differentiated cells with branching neurites in the population of differentiated cells. (d) Percentage of aligned neurites as calculated from each analyzed image of the differentiated cells. Statistical significance is represented with an * for P < 0.05.

As stated in the Introduction, patterns with a low L/D ratio are preferred for better alignment. When height is varied for patterns with similar L/D ratios, instead of similar lateral dimensions, it can be observed whether in this case also differentiation and neurite length are influenced but not alignment.

1. Height variations at high ridge to period ratios

For the first comparison between patterns of similar L/D ratios and similar heights, D600L230, D800L400, and D1000L500 were used (Fig. 7). The mean L/D = 0.52 and height = 99.5 nm for process I were compared to a mean L/D = 0.59 and height = 123.3 nm for process II.

The differentiation of cells showed a statistically significant increase at P < 0.01, with 0.25 ± 0.12 for process I and 0.42 ± 0.08 for process II. Neurite length did not increase significantly with a value of 45.15 ± 13.0 nm for process I and 51.7 ± 12.8 nm for process II. Branching did not increase significantly with a value of 0.02 ± 0.03 for process I and 0.03 ± 0.06 for process II. Neurite alignment showed an increase from 76.7 ± 18.7% for process I to 80.4 ± 14.5% for process II. From these data, the increase in height had no significant impact on neurite alignment. However, it was clear that differentiation was increased and that neurite length and branching followed a similar trend, but this was not statistically significant.

2. Nanogroove period variations at a high ridge to period ratio

While height was the parameter that was explicitly different between processes I and II, the lateral dimensions also influenced the neuronal cell culture and were compared, in particular the lateral dimensions that made up the L/D ratio. As such, it is useful to compare nanogrooves for similar L/D ratios and heights and study the effect of the pattern period (Fig. 8). For this comparison, four groups were selected: group D300 (D300L150 and D300L180 from process I), D600 (D600L230 from process II), D800 (D800L400 from process I), and D1000 (D1000L230 from process I), where the mean L/D = 0.59 and H = 104.2 nm. AFM measurements on group D300 did not show a height that was measured fully and therefore were only used for qualitative comparison.

Differentiation was 0.23 ± 0.10 for D300, 0.43 ± 0.13 for D600, 0.20 ± 0.14 for D800, and 0.24 ± 0.10 for D1000. A statistically significant difference was observed between D300 and D600 at P < 0.05. Neurite length did not increase significantly between groups, with values of 46.2 ± 12.8 nm for D300, 51.9 ± 6.5 nm for D600, 44.7 ± 9.2 nm for D800, and 39.3 ± 8.6 nm for D1000. However, as with differentiation, values were higher for the D600 group. Branching showed values of 0 ± 0.02 for D300, 0.03 ± 0.04 for D600, 0.02 ± 0.01 for D800, and 0.03 ± 0.03 for D1000. Here too, values were higher for group D600 compared to the other groups. Neurite alignment was similar for all groups with no statistically significant differences. Alignment values were 70.1 ± 17.9% for D300, 78.9 ± 19.7% for D600, 67.3 ± 8.9% for D800, and 72.5 ± 16.7% for D1000. These results show that in particular, pattern period D = 600 nm aided in differentiation but not in alignment. Group D300 seemed to perform similarly with regard to differentiation, neurite length, and branching compared to groups D800 and D1000. Despite the qualitative comparison of the D300 group with the other groups, this aided in observing these properties and inferred that pattern height may have been similar compared to the other samples. This observation would need to be confirmed using an advanced pattern measurement method. Alignment for group D300 is similar in median and IQR value as the other groups; however, the total spread as shown by the whiskers revealed that the pattern may not function as robustly with regard to aligning neurites. Likely, the distance between the ridges was too small and neurites bridged the grooves between the ridges as if the substrate surface was flat.

3. Nanogroove period variations at a low ridge to period ratio

Pattern period D = 600 nm showed improved differentiation at lower L/D ratios. Thus, it is important to also study whether this pattern period has an effect compared to patterns of similar L/D ratios and similar heights (Fig. 9). For
this comparison, three groups were selected: group D600 (D600L180 from process I), group D800 (D800L230 from process I), and group D1000 (D1000L230 from process I), where the mean L/D = 0.38 and H = 99.4 nm (Fig. 9).

Differentiation was 0.33 ± 0.10 for D600, 0.20 ± 0.19 for D800, and 0.38 ± 0.13 for D1000 with no statistically significant differences. Neurite length did not increase significantly between groups, with values of 46.7 ± 7.0 nm for D600, 37.9 ± 12.5 nm for D800, and 51.7 ± 21.4 nm for D1000. Branching showed values of 0.00 ± 0.01 for D600, 0.01 ± 0.03 for D800, and 0.01 ± 0.02 for D1000 without any statistically significant differences. Neurite alignment was 87.1 ± 9.3% for D600, 72.0 ± 14.1% for D800, and 85.5 ± 8.0% for D1000. A statistically significant difference was observed between groups D600 and D800. These results show that the significance of the nanotopographical cues from the actual lateral dimensions was reduced and the influence of the L/D ratio was higher at low L/D values. Differentiation, neurite length, and branching were not significantly influenced by lateral dimensions; however, the alignment was decreased specifically in the case of group D800. Considering that both groups D600 and D1000 scored higher, a shift in the mechanisms behind the topographical cues of the patterns may be seen here, as D1000 patterns can be considered microgrooves of small size. At D600, neuronal cells stayed on top of the ridges, whereas at D1000, grooves may become wide enough for cells to attach to both the ridge and the groove of the pattern. To provide detailed information on this phenomenon, additional experiments are required, e.g., characterizing neurite morphology by scanning electron microscopy (SEM) or AFM. 

IV. SUMMARY AND CONCLUSIONS

In conclusion, we investigated the effect on neuronal properties of nanogrooved PDMS substrates fabricated via two different fabrication processes: a J-FIL resist pattern on a polished silicon wafer and an RIE all-silicon mold, which was fabricated using the same J-FIL resist pattern as a mask and used in parallel to obtain COC templates through thermal nanoimprinting. Soft lithography of PDMS using the pattern. To provide detailed information on this phenomenon, additional experiments are required, e.g., characterizing neurite morphology by scanning electron microscopy (SEM) or AFM.

A ridge to period ratio of <0.4 resulted in significantly lower neurite alignment. It is suggested here that topographical cues may shift from nanoscale to microscale cues. Follow-up studies using high-resolution analysis of neurites by means of confocal microscopy, AFM, and SEM may help in further specifying how neurites interact with their physical environment.

The knowledge obtained in this work can be used as the basis for optimization in the bottom-up engineering of neuronal network architecture. This aids the design of in vitro assay systems for BOC applications and may help to understand the relationship between form and function within a neuronal network.

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33 See supplementary material at https://doi.org/10.1116/1.5048069 for a complete table of measured dimensions for each of the 27 nanogroove patterns in PDMS substrates as derived from processes I and II.