PC12 polarity on biopolymer nanogratings

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Abstract. Cell differentiation properties are strongly entangled with the morphology and physical properties of the extracellular environment. A complete understanding of this interaction needs artificial scaffolds with controlled nano-/micro-topography. We induced specific topographies by nanoimprint lithography (NIL) on tissue culture polystyrene (TCPS) dishes substrates and, using light microscopy and high-magnification scanning-electron-microscopy, quantitatively compared the changes in PC12 differentiation phenotype induced by the periodicity of the nanopatterns. This analysis revealed that nanogratings reduce the number of neurites produced by PC12 cells upon treatment with NGF and that neuronal bipolarity correlated with an increased stretching of the cell body and a reduced length of the cell neuronal protrusions.

1. Introduction
The study of neuron differentiation and neurite outgrowth mechanisms is of primary relevance in view of their role in neuronal tissue development and of their applications in biomedical engineering and biomimetics [1]. Cell morphogenesis, differentiation and adhesion properties are strongly intermingled with the morphology and composition of the extracellular environment through complex interactions between cells and a plethora of external physical and chemical stimuli [2, 3].

A complete understanding of these interactions necessitates artificial scaffolds with controlled nano-/micro-topography where environmental properties can be independently tuned to reveal their effect on cell differentiation. Realization of controlled micro-topography on bio-compatible substrates proved to be difficult owing to the typical incompatibility of these materials with standard micro-fabrication techniques. Only recently, non-conventional approaches (like soft lithography, imprint lithography and laser ablation) [4] allowed good versatility and reliability in biopolymer high-resolution patterning.

Tissue culture polystyrene (TCPS) dishes were chosen as patterning substrates and the desired topography was induced by nanoimprint lithography (NIL) without surface chemical functionalization. On these substrates, we could recently demonstrate guided PC12 differentiation [5]. Using light microscopy and high-magnification scanning-electron-microscopy, here we quantitatively compared the changes in PC12 differentiation phenotype induced by the periodicity of nanogratings fabricated on TCPS substrates.
2. Materials and methods

2.1. Substrate fabrication
We fabricated sub-micrometer patterns on polystyrene (PS) substrates by NIL. The molds were obtained by electron-beam lithography (EBL) and reactive-ion etching (RIE) starting from commercial p-doped silicon wafers (SYLTRONIX, France).

TCPS commercial plates were imprinted using an Obducat Nanoimprint 24 system. After cleaning with 2-propanol, TCPS were placed on top of the mold and softened by increasing the temperature up to 120 ºC. Afterwards, a pressure of 20 bar was applied for 5 minutes before cooling down to 70 ºC, i.e. well below the glass transition temperature of PS (Tg = 95-105 ºC). Finally the pressure was released and mold and TCPS detached. The topography of the imprinted gratings was systematically analyzed by atomic-force-microscopy before proceeding with cell culturing.

2.2. Image and Statistical Analysis
After 48 h of NGF treatment, the cells were fixed with 2.5% glutaraldehyde in sodium cacodylate buffer (pH 7.2, 0.15 M) for 1h and successively rinsed three times with sodium cacodylate buffer. Samples were then dehydrated with ethanol absolute at different concentrations: 50%, 70%, and 98 % [6]. Images were collected using standard optical-microscopy and digital image-acquisition.

Collected images where converted into 8-bit grayscale TIF files using ImageJ (National Institute of Health, USA) software and loaded into NeuronJ [7] plug-in of ImageJ. We restricted our analysis to cell protrusions with measured length equal or bigger than the cell body thickness. Cell body thickness was measured with ImageJ in a region of the cell not containing neurites. The beginning of a neurite was defined as follows. First, the central thickness of individual cell protrusions (cnt) was measured in a central region of the neurite where it appeared constant. The neurite thickness measure was then repeated in regions closer to the cell body. The point in which the thickness reached twice the value of cnt was defined as the beginning of the neurite. Cell body perimeter, area and circularity were measured manually outlining the cell body contour taking into account the previous definition of neurite beginning points. Mean values and standard errors were calculated for all the parameters.

Statistical comparison of measured neurite lengths and cell body area, perimeter, and circularity on differently patterned and flat surfaces, was performed with a two tailed Mann-Whitney test (α=0.05). Statistical comparison of neurite number in cell grown on patterned and flat surface was performed with a Chi-square test. For each culturing condition, cells were grouped into 5 categories depending on the number of neurites (0, 1, 2, 3, 4 or more) and a p value was calculated from the Chi-square distribution (degrees of freedom = 4).

All parameters were measured in individual cells from at least three independent specimens.

2.3. SEM imaging
After 6 h of NGF treatment, the cells were fixed as described in Sec. 2.2. and coated with a 20-nm-thick gold layer by thermal evaporation. The metal layer was shorted to the SEM sample holder to allow proper electron discharge during imaging. The substrates were then loaded into a LEO 1525 field emission SEM and image acquisition was carried out by secondary-electron detection with the Everheart-Thornley detector in order to enhance the topography of cell-substrate interfaces.

3. Results and discussion
PC12 cells were plated on TCPS substrates characterized by different dimensions of the imprinted patterns. Gratings with 50% duty cycle, 200-nm-depth and linewidth of 500 nm (Type-1) and 750 nm (Type-2) were used for this study. As we already demonstrated [5], this culturing condition determines highly non-isotropic PC12 differentiation, leading almost all the axons (exceeding 90%) aligned within 30º to Type-1 gratings, and the 75% of axons aligned within 30º to Type-2 gratings.

These three specific topographic stimuli led to different PC12 phenotypes, characterized by peculiar properties of polarity and body shape. Top panels of Figure 1 show light-microscopy images.
of three typical cells, relative to populations cultured on Type-1 (a), Type-2 (b) gratings and on flat substrates (c). In addition to the expected axon alignment along Type-1 and Type-2 geometries, reduced cell-body circularity and polarity with respect to the control-substrate case are revealed.

This preliminary visual characterization was supported by a complete statistical analysis. The histogram reported in Figure 1d depicts the percentage of cells exhibiting 0, 1, 2, 3, 4 or more neurites respectively as measured on Type-1 gratings (light grey), Type-2 gratings (black), and flat surface (grey). In case of Type-1 and Type-2 patterns, the most probable number of neurites per cell was 2, whereas in case of flat surface, the highest probability was found for the case of 3 neurites per cell. The distribution obtained for flat surfaces was also significantly different from those obtained for nanopatterns. In fact, in these cases the distributions showed a pronounced cut-off at 2 neurites per cell with negligible probability to find more than 2 neurite per cell.

Figure 1: PC12 differentiation on patterned surfaces. Typical cell phenotype after 48h NGF treatment on Type-1 grating (a), Type-2 grating (b), and flat surface (c). (d) Distribution of the number of neurites per cell. Histogram height individuates the percentage of cells showing 0, 1, 2, 3, 4 or more neurites respectively as measured on Type-1 gratings (light grey), Type-2 gratings (black); and flat surface (grey). p values of Chi-square tests report about statistical significant differences. (e) Average neurite length. Neurite lengths are reported as 10-90 percentile distribution. The population mean is reported as a horizontal black line enclosed in a rectangular box whose lengths represent the standard error of the mean. p values of Mann-Whitney tests report about statistical significant differences.

Inhibition of multipolarity was found to be associated with reduced neurite development (Figure 1e). The mean neurite length measured on flat substrates was 21 ± 2 µm and very similar value was obtained for cells on Type-B gratings (19.4 ± 2.4 µm). Instead, in case of Type-1 gratings the mean neurite length was reduced to 13.4 ± 0.7 µm, and this inhibition was strongly confirmed by the low p-value (0.0001) obtained from the Mann-Whitney test.

Finally, we analyzed cell-body area, perimeter and circularity. The data showed almost unchanged body areas (102 ± 7 µm², 107 ± 9 µm² and 105 ± 15 µm² for Type-1, Type-2 gratings and flat surfaces, respectively) but different body elongations. Cells cultured on Type-1 gratings were characterized by mean perimeter and circularity of 46 ± 2 µm and 0.61 ± 0.02 respectively, less than what measured for Type-2 gratings (42 ± 2 µm and 0.75 ± 0.02) and for flat substrates (40 ± 3 µm and 0.80 ± 0.03). Mann-Whitney tests returned p = 0.04 and p = 0.0001 for perimeter and circularity comparison between cell populations cultured on Type-1 gratings and on flat surfaces, and p = 0.9 and p = 0.2 for the same comparison between Type-2 and flat.

Axon outgrowth direction and body morphology changes result from the local reorganization of the cell cytoskeleton. Environmental stimuli have been proven capable of inducing such cellular responses, but the mechanism by which the cells locally integrate these information remains largely
unexplored. A crucial role is played by the dynamics sensing of filopodia and lamellipodia at the growth cone during axonal protrusion [8]. The dynamics of actin polymerization at this location might be strongly affected by the presence of regular nanopatterns, leading to preferential stability in one direction owing to the substrate broken symmetry.

**Figure 2** shows high-magnification SEM images of a growth cone (left) and an axon (right) protruding from a PC12 cell. These demonstrate that filopodia and lamellipodia explore both depressed and elevated regions spreading without apparent directionality. Nonetheless, there is experimental evidence that axon guidance happens on top of nanoridges [6]. One could envision that axon guidance results from the spatial variation of actin cytoskeleton stability at the growth cone. Reduced persistence of the actin protrusions crossing the periodic patterning and/or higher stability of those oriented along the grating may guide the processes of axon engorgement and consolidation leading to neurite outgrowth. For this reason it will be important to perform studies of actin dynamics on patterned surfaces with high temporal and spatial resolution.

![Figure 2](image_url)

**Figure 2:** SEM images of PC12 cells differentiating on nanopatterned substrates: details of sprouting filopodia and lamellipodia.

### 4. Conclusions

This analysis revealed that nanogratings reduced the number of neurites produced by PC12 cells upon treatment with NGF. Neuronal bipolarity was correlated with an increased stretching of the cell body and a reduced length of the cell protrusions. Altogether our data indicate the presence of an inhibitory signal resulting from the cellular interaction with the patterned surfaces. This signal may coordinately block the production of out-of-guide neurites and hamper the outgrowth of the guided ones.

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