1.3.7 Nanotechnology for guided cell differentiation

The morphogenesis of mammalian organs and tissues relies on the ability of individual cells to respond to a vast range of extracellular signals. Among these are gradients of soluble molecules such as growth factor and cell-secreted mitogens encoding for preferential directions over long distances. A second class of signals is provided by the chemical and physical properties of the extracellular matrix (ECM) in the cell proximity. These guidance cues are read by cells in a process that requires the activity of specific cell-adhesion machinery and leads to the remodeling of cell shape. Several cellular responses such as polarization, migration, proliferation, and apoptosis are elicited by a direct cell-ECM interaction in virtually all cell types. A pivotal role in this process is played by the substrate nanotopography. In order to unravel the mechanisms by which differentiating cells read the local topography it is crucial to decouple its effects from those stemming from other chemical and physical stimuli. Thanks to recent advances in biomaterial nanofabrication, selected morphological aspects of the ECM are now reproducible in vitro through the realization of artificial scaffolds with controlled topography (Fig. 1). The related research activity at NEST lab follows two strategies: (i) improving the chemical and morphological properties of biomimetic two-dimensional (2D) scaffolds with nanostructured surface modifications (ii) understanding the cellular mechanisms regulating topographical guidance (iii) evaluate the impact of "noisy" guidance and investigate ways to enhance resistance to such "noise".

We established successful protocols to fabricate anisotropic nano-textures (i.e. nanogratings, alternating lines of grooves and ridges of submicron size) on thermoplastic materials. Particular attention was paid to the biocompatibility of the resulting cell scaffold. To this aim tissue-culture polystyrene (TCPS) was initially chosen [1]. The desired topography could be induced by nanoimprint lithography (NIL) [1, 2]. No further surface chemical functionalization was performed (Fig. 1A) and the resulting imprinted substrates proved optimal for the growth of PC12 cells, a well-established model of neuronal differentiation [3]. Cell-nanograting interaction on TCPS substrates was investigated specifically focusing on two aspects: the efficiency of neurite alignment (Fig. 1B) and cell polarity state (Fig. 1C-E) as induced by nanogratings with varying geometries [1, 2]. As previously reported for other cell types, PC12 cells were only marginally affected by larger structures (>1 μm linewidth) whereas sub-micron topography led to highly increased non-isotropic differentiation. Figure 1 displays the degree of axon outgrowth control determined by 750 and 500 nm linewidth gratings. The latter was found to be more effective, leading to over 90% of the axons aligning within 3 degrees to the gratings, and almost 70% of axons aligned within 10 degrees. For what concerns 750 nm linewidth substrates, 75% of axons were aligned within 30 degrees and the 36% within 10. For comparison, the same analysis was performed on cells differentiating on flat, unpatterned TCPS where no preferential directionality was observed (Fig. 1B).

Nanogratings reduced to two the number of neurites produced by PC12 cells upon treatment with NGF (Fig. 1D). Neuronal bipolarity was correlated with an increased stretching of the cell body and a reduced length of the cell protrusions (Fig. 1E).

We next analyzed the mechanisms governing the interaction between differentiating neuronal cells and our nanoimprinted topographies [4, 5]. Our work aimed at defining a biological link between the topographical configuration

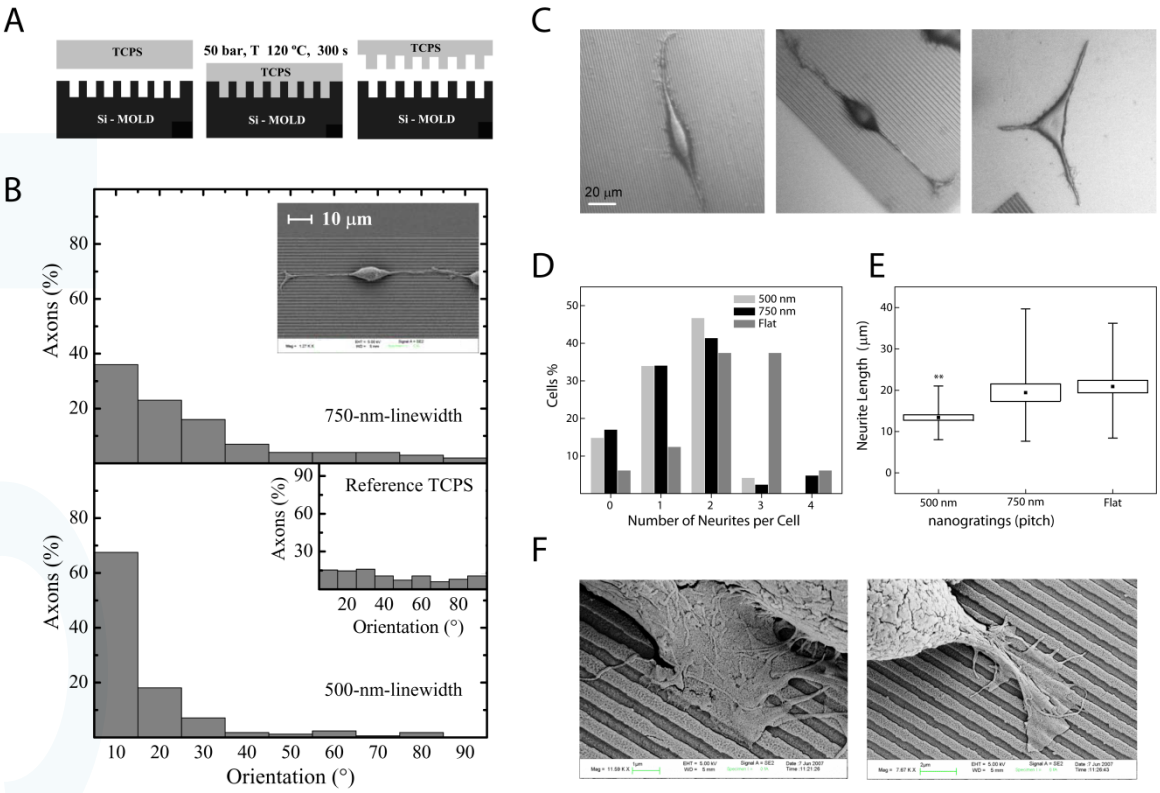

Figure 1. A) Fabrication process of TCPS-nanostructures and their characterization. TCPS were placed on top of the silicon mold and softened by increasing the temperature up to 120 ◦C (left). 20 bar pressure was applied for 5 min before cooling down to 70 ◦C (middle). The pressure was finally released and mold and TCPS detached (right). B) Quantitative analysis of guided axon outgrowth on TCPS nanogratings: percentage of axons as a function of the angle between the axon and grating-line directions for 750 nm (upper panel) and 500 nm (lower panel) linewidth gratings. The same analysis is reported in the inset of the lower panel for cell differentiation on flat TCPS. Inset of upper panel: scanning electron microscope image of a PC12 cell on a nanograting showing bipolar conformation, enhanced body elongation and guided axon outgrowth. C) Typical cell phenotype on 500 nm grating (left), 750 nm grating (center), and flat surface (right). D) Distribution of the number of neurites per cell. E) Average neurite length. Neurite lengths are reported as 10-90 percentile distribution. F) SEM images of PC12 cells differentiating on nanopatterned substrates: details of sprouting filopodia and lamellipodia.

of the substrate and the resulting cell polarity. For this activity we evaluated the use of new biocompatible materials with enhanced optical properties allowing the use of high-resolution live-cell microscopy in physiological conditions [6]. In this direction the nanoimprint protocol defined for TCPS could be further developed to obtain similar nanogratings imprinted on COC. Using these improved substrates we revealed the role played by integrin-based adhesion complexes, the focal adhesions, during neuronal differentiation on anisotropic nanotopographies [4]. First, we showed that during neuronal differentiation, topographical anisotropies control focal-adhesion maturation. Modulation of adhesions was the driving mechanism that selected the properly aligned neurites.

Notably, by varying a single topographical parameter of the substrate, orientation and maturation of focal adhesions could be finely modulated yielding independent control over the final number and the outgrowth direction of neurites [7]. Second, we demonstrated that a guiding topography can induce and consolidate a single polarity state by regulating the cell differentiation machinery. Hence, differentiating neurons can not only 'read' the topographical guidance cues, but can also re-localize specific cellular activities along preferential directions and thus 'learn' to achieve a committed differentiation.

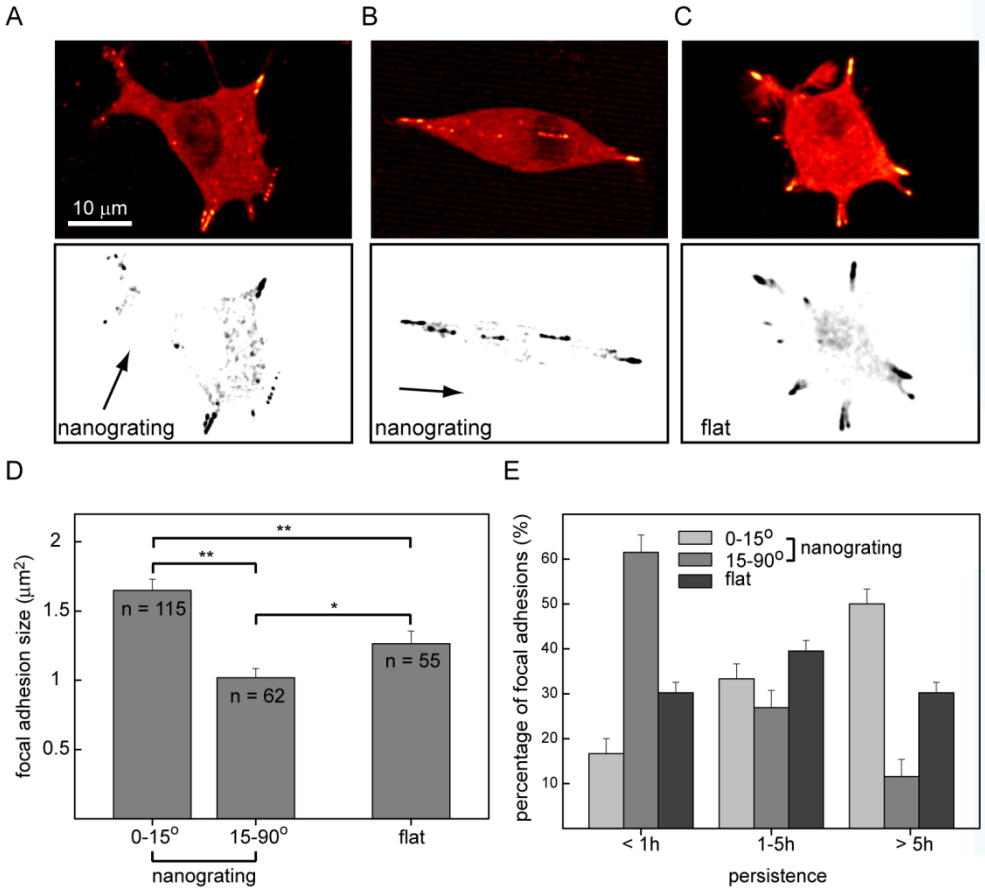

Figure 2. Angular modulation of focal-adhesion size and persistence. **A**) and **B**) Distribution of paxillin-EGFP fluorescent signal in PC12 cells differentiating on nanogratings, and [**C**)] on flat substrates. Statistical comparison of focal adhesion size **D**) and persistence **E**) on nanogratings and on flat substrates.

Moreover, we addressed the effect of nanogratings on the migration properties of differentiating PC12 cells and correlated their behavior with the polarity state induced by the substrate. During neuronal differentiation, cell-substrate interaction is sufficient to induce directional migration along the nanogratings (Fig. 3). Control cells contacting flat substrates migrated freely in all directions while cells differentiating on nanogratings showed slower migration characterized by an angular restriction that confined cell movements. Finally, we

showed that directional migration on nanogratings is linked to a specific organization of the cell cytoskeleton reflecting the nanograting directionality [8].

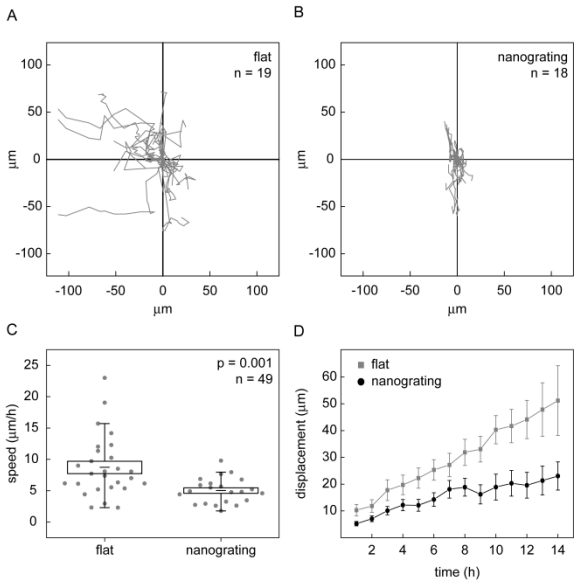

Figure 3. Migration of PC12 cells differentiating on nanogratings or on flat substrates. **A)** Individual trajectories of PC12 cells (n=19) migrating on flat substrate. **B**) Individual trajectories of PC12 cells (n=18) migrating on nanogratings. For each trajectory (gray lines in panels A and B) the initial cell position was translated into the origin of the graph. **C**) Statistical analysis of cell speed during migration on nanogratings and flat substrates. The 5 to 95 percentile distribution (vertical line) was derived from individual data points (gray circles). The population mean is reported as a horizontal black line enclosed in a rectangular box whose length represents the standard error of the mean. The reported p-value was obtained performing a Mann-Whitney test. For this analysis 49 cells were considered. **D**) Cell displacement during migration. The average distance traveled from the origin is reported for cells migrating on flat substrates (gray squares) and on nanogratings (black circles) for each time of measure (_t = 1 h), for a total of 14 hours.

We also studied neuronal cell response to directional stimuli by exploiting nanogratings with a controlled amount of random nanotopographical noise [9]. We showed that the loss of neurite guidance is not linear with noise, but exhibits a threshold effect, correlating with changes in FA maturation and spatial organization. Remarkably, by acting pharmacologically on cell contractility, we could promote aligned FA maturation, and improve neurite-guidance tolerance to noise [10].

Finally, the human neuroblastoma cell line SH-SY5Y was interfaced with plastic nanogratings and contact guidance was investigated under proliferating conditions and upon differentiation after treatment with retinoic acid (RA) and brain-derived neurotrophin factor (BDNF), and compared with mouse primary hippocampal neurons (HNs). RA/BDNF improved SH-SY5Y responsiveness to NG directional cues, and significantly enhanced the alignment to the nanograting lines. HNs behaved similarly, showing a marked change in network organization if cultured on NGs [11].

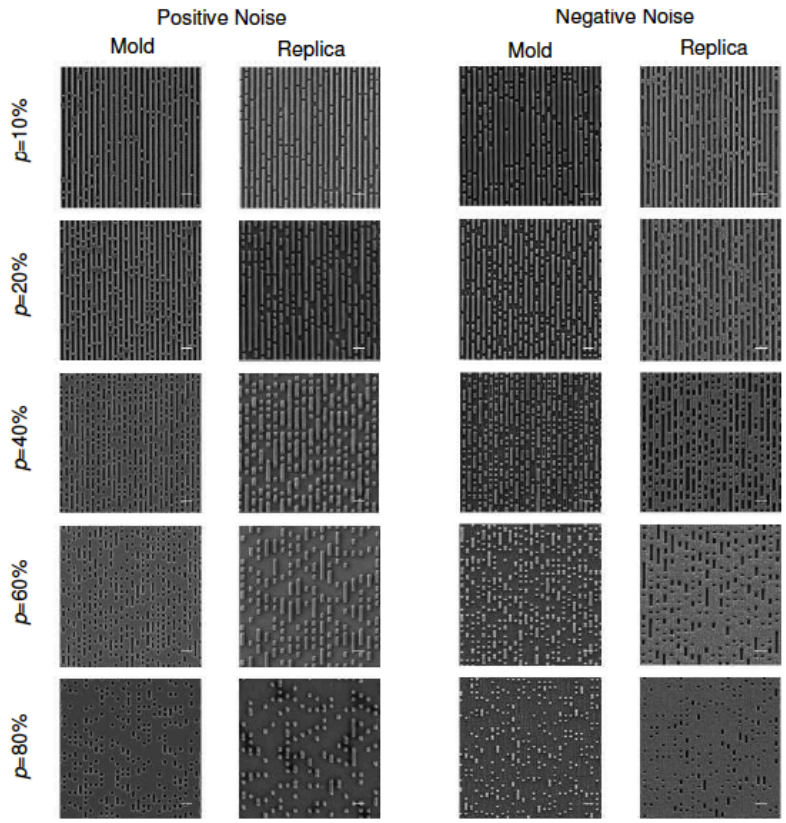

Figure 4. Scanning electron microscope images of the noisy molds and replicas. p indicates the noise level. Scale bars = 2 μm

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