Increasing nanogroove height enhances neuron outgrowth alignment

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Nanogroove topography has been demonstrated to stimulate the alignment of cell outgrowths providing defined cell-cell interactions in neuronal networks as well as potentiating stem cells differentiation into the neuronal lineage[1,2,3]. Here, our new findings detail the effect of height variations occurring during reactive ion etching (RIE) in the pattern transfer of jet and flash imprint lithography (J-FIL) resist to a durable silicon template (Fig.1A). Results show that a ~23 nm increase in height can pronounce alignment.

We copied the original resist pattern (*i*) and the silicon template (*ii*) into cyclic olefin copolymer (COC) working molds (*iii*) and (*iv*), respectively (Fig.1B). From molds *iii* and *iv*, the final nanogrooved cell culture substrates, (*v*) and (*vi*) respectively, were made by soft lithography of polydimethylsiloxane (PDMS) (Fig.1C). We analyzed geometrical differences by atomic force microscopy (AFM). Neuronal cultures using SH-SY5Y cells were performed, fixed and stained at DIV21. Subsequently, fluorescence images (Fig.3A) were collected for the application of an in-house automated screening algorithm detecting cell bodies and outgrowths[4]. By means of this algorithm, the level of outgrowth alignment with respect to the direction of the nanogrooves was determined.

For each of the total of 27 tested nanogrooved PDMS patterns, defined by ridge width (L) and pattern period (D), similar pattern ridge width to period ratios (L/D) are retained for both *v* and *vi* as confirmed by AFM (Fig.2A). However, for patterns where groove bottoms were detectable by AFM, we revealed an average increase of pattern height (H) of ~23 nm due to the etching process required for *ii* (Fig.2B). This increase in height also correlates with a ~10% rise in outgrowth alignment (Fig.3B). In conclusion, both L/D and L/H ratio enhance neural outgrowth alignment and hence define neuronal network architecture.
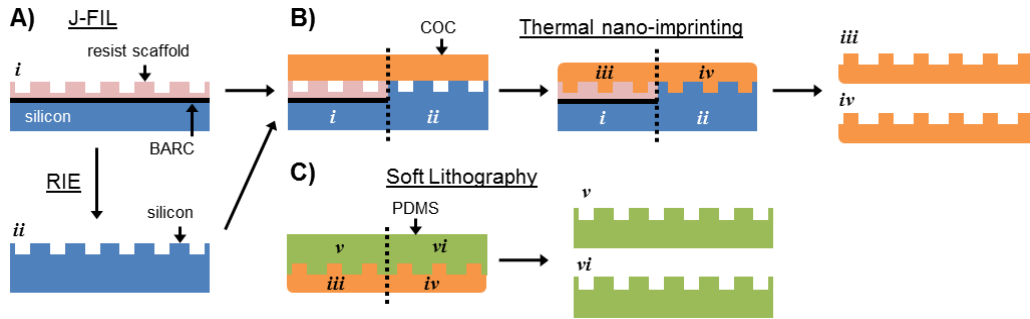
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Figure 1: Nanogrooved substrate fabrication. (A) Jet and flash imprint lithography (J-FIL) resist (*i*), can be transferred into silicon by reactive ion etching (RIE) (*ii*). (B) Using *i* and *ii* as nano-imprint templates for pattern transfer into cyclic olefin copolymer (COC) results in molds *iii* and *iv* respectively. (C) Soft lithography of polydimethylsiloxane (PDMS) is used to transfer patterns from *iii* and *iv* to the final PDMS cell culture substrates, *v* and *vi* respectively.
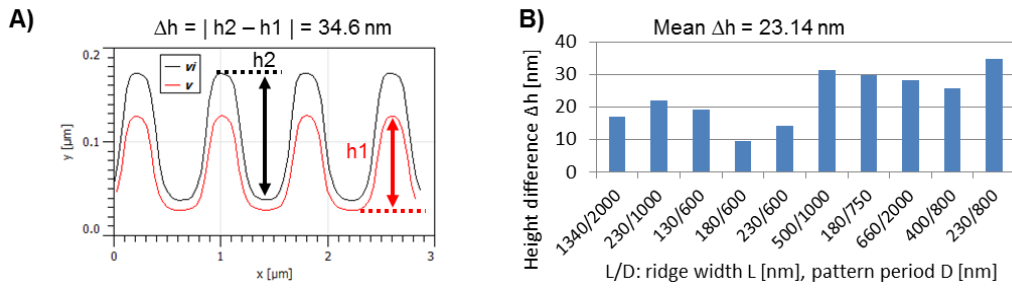
Figure 2: Nanogrooved patterns measured with atomic force microscopy (AFM). (A) Example of a line profile for ridge width (L) of 230 nm and pattern period (D) of 800 nm for polydimethylsiloxane substrates *v* and *vi* (Fig.1). Whilst L/D remains similar, height is increased for *vi* (h2) compared to *v* (h1) by 34.6 nm. (B) Height difference, as measured in (A), for several nanogrooved patterns with varying L/D. The mean pattern height is increased by 23.14 nm for *vi* compared to *v*.
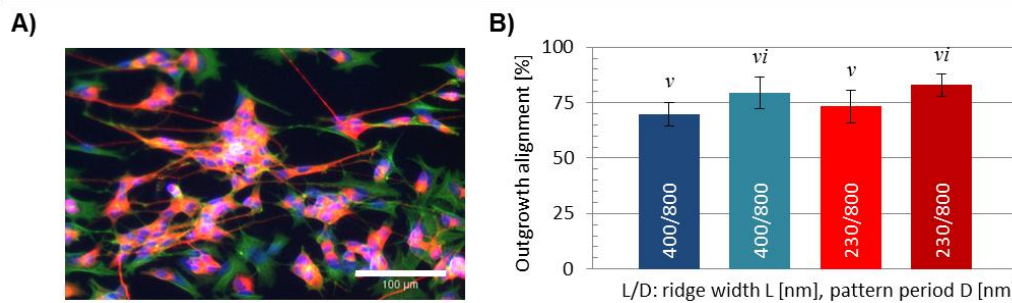
Figure 3: Outgrowth alignment to underlying patterns. (A) Example image of neuronal cells on a nanogrooved pattern with ridge width (L) of 230 nm and pattern period (D) of 800 nm. Red shows β-Tubulin III, green shows actin and blue cell nuclei. Scale bar is 100 μm. (B) Outgrowth alignment for cells on patterns from either polydimethylsiloxane substrate *v* or *vi* (Fig.1). Mean outgrowth alignment increases by ~10% for *vi* compared to *v*. Pattern dimensions are detailed using L/D.