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Low Cost, Patterning of Human hNT Brain Cells on Parylene-C with UV & IR Laser Machining

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Abstract— **This paper describes the use of 800nm femtosecond infrared (IR) and 248nm nanosecond ultraviolet (UV) laser radiation in performing ablative micromachining of parylene-C on SiO_2 substrates for the patterning of human hNT astrocytes. Results are presented that support the validity of using IR laser ablative micromachining for patterning human hNT astrocytes cells while UV laser radiation produces photo-oxidation of the parylene-C and destroys cell patterning. The findings demonstrate how IR laser ablative micromachining of parylene-C on SiO_2 substrates can offer a low cost, accessible alternative for rapid prototyping, high yield cell patterning.**

I. Introduction

The field of cell patterning is concerned with controlling the growth and proliferation of cells in a reliable and robust manner. To date cell patterning has exploited a variety of techniques, such as micro-contact stamping, physical immobilisation, photo-lithographic techniques [1–4]. While these processing methods may be a viable method for mass production of the same chip design, for example, photolithographic masks can be reused, the costs involved may be a limiting factor in a research environment where many experimental designs are required. The motivation for this work was therefore to apply infrared (IR) and ultraviolet (UV) polymer ablation techniques to established parylene-C (Poly-monochloro-para-xylylene) and SiO_2 substrates for cell patterning. Previous work by Delivopoulos et al. [5, 20] and Unsworth et al. [6–8] using photolithographic techniques to pattern parylene-C on SiO_2 has demonstrated patterning of both rodent and human hNT neurons and glia. The parylene-C/SiO_2 substrate used required complex manufacturing steps including photolithographic techniques combined with a dry etch to remove the chemically inert parylene-C.

Laser based micromachining of materials has been used to pattern cells *in vitro*, reviewed in [21] for cellular micro-arrays [22,23], cell based assays [24] , microfluidics [25] and for creating 3D scaffolds [26].

UV Laser ablation of organic polymers is a long standing research field [9] and has been applied to parylene-C in different contexts [10], [11] but its application to cell patterning has not been investigated. One reason for this is due to the fact that parylene-C is known to oxidize under UV light resulting in the formation of aldehyde and carboxyl groups at its surface [12–14]. This photo-oxidation has been shown to reduce the conformity of neuronal and glial cells to the underlying pattern [14]. In contrast although femtosecond IR laser ablation has been applied to other organic polymers [15], [16], IR laser ablation of parylene-C has not been reported. Thus, the work reported in this article will be the first application of laser ablation to assist in the patterning of biological cells on parylene-C.

The objectives of this work were to first assess the viability of IR and UV laser ablation, as a method for accurately creating parylene-C patterns. Subsequently, we assess the conformity of human hNT astrocytes on underlying parylene-C patterns.

II. Materials & Methods

A. Fabrication of Parylene-C on SiO_2 Substrates

Initially, silicon wafers were oxidized in a furnace (H_2 1.88 sccm and O_2 1.25 sccm) at 950^{o}C for 40 min to produce a 200nm SiO_2 layer (measured with a Nanometrics NanoSpec/AFT, Microarea gauge). Parylene-C of 100nm was then deposited at room temperature on the oxidized wafers at a rate of 1.298nm per mg of dimer using a Labcoter 2 deposition Unit (Model PDS2010).

B. Laser Ablation of Parylene-C

UV laser ablation was performed using a Coherent Xantos XS 248nm UV KrF excimer laser with a pulse duration of 10ns, a repetition rate of 500Hz and laser fluences of 80 – 250 mJcm^{-2}. IR laser ablation was performed with a Coherent Legend Elite 800nm IR laser with a pulse duration of 110fs, a repetition rate of 500Hz. IR lasering was performed using single pulse high power

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ablation at 684mJcm^{-2}. The beams of both lasers were masked to produce 50x50µm square ablations on the parylene-C surface and a moving computer controlled machining stage ablated the desired patterns with a pulse overlap of 5µm. The patterned chips contained forty, 1000um long strips of parylene-C with widths of 5, 10 and 20µm at a spacing of 140µm (Figure 1(i), highlights the parylene-C strips that were retained after the laser micromachining of the material).

C. Stem Cell Culture & Microscopy

An NTera2/D1 stem cell line, purchased from the American Tissue Culture Collection (ATCC; CRL-1973), was differentiated over 10 weeks according to the protocol developed in [7]. Cells seeded on the patterned substrate at ~100 cells/mm^2 and allowed to migrate to the patterns over a 6 day period.

D. Labelling, Mounting and Imaging

The anti-vimentin antibody (Abcam; ab15248) was used to stain the permeabilised astrocytes. Primary staining was conducted at 1:1000 and detected using anti-rabbit Alexa 488 (Invitrogen; 1:400). The hNT astrocyte nuclei were stained using Hoechst 33258. The chips were mounted using AF1 (a 50:50 Phosphate buffered saline) PBS:glycerol mix (Citifluor).

Cell images were taken with an Olympus BX53 fluorescence microscope using 4x and 10x objectives. Hoechst stained nuclei were visualised using the 350/360nm argon laser and Alexa 488 staining was visualised a 488nm argon laser was used. The laser was also used in reflection mode in order to visualise the parylene-C strips. For data analysis a ×4 objective gave good resolution of the 8mm^2 chips. In addition, a ×10 objective was employed for detailed observation of the quality of the cell patterning on the ablated parylene-C surface.

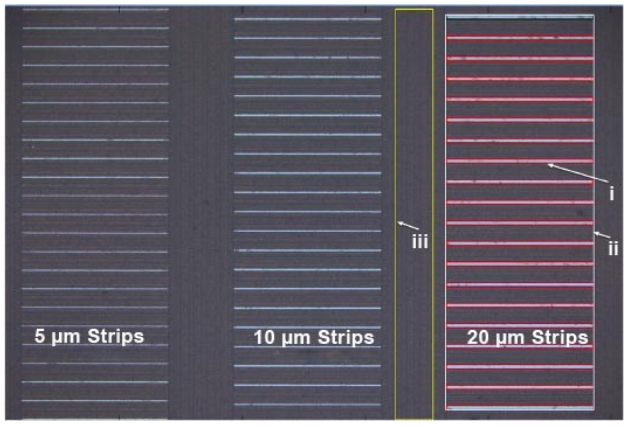

Figure 1. One half of a laser ablated chip. Cell coverage was measured on 3 Regions of Interest (ROI); 'on parylene'(regions in red (i)), 'adjacent to parylene'(region in white but not in red, (ii)) and 'off parylene' (region in yellow, (iii)). The parylene-C strips were 1000µm in length, 100nm thick and had a strip spacing of 140µm .

E. Image Processing

Image pre-processing involved image alignment, histogram equalisation and application of a 5x5 Weiner filter to remove noise effects introduced by the histogram equalisation. Cellular content was identified using the total pixel count of a threshold image and was normalized by the area of the Region of Interest (ROI). The percentage of the substrate covered by cells was measured for three regions, 'on the parylene-C', 'adjacent to the parylene-C' and 'off the parylene-C,' as shown in Figure 1. A more detailed description of the image pre-processing steps can be found in Unsworth et al [8].

III. Results

A. UV laser ablative patterning

Here we discuss the use of UV laser ablation in micro machining substrates for cell patterning. Visual examination of the micro machined substrates revealed no visible damage to the parylene-C strips and no damage to the underlying SiO_2 substrate. This is consistent with existing literature relating to UV laser ablation of parylene-C [10], [11].

The hNT neuronal coverage for UV ablated parylene-C/SiO2 substrates was investigated for the strip widths and pulse fluences described above. In all cases, the UV ablated substrate was found not to promote cell patterning. Figure 2(a-b) highlights typical non-patterned cell cultures that were observed.

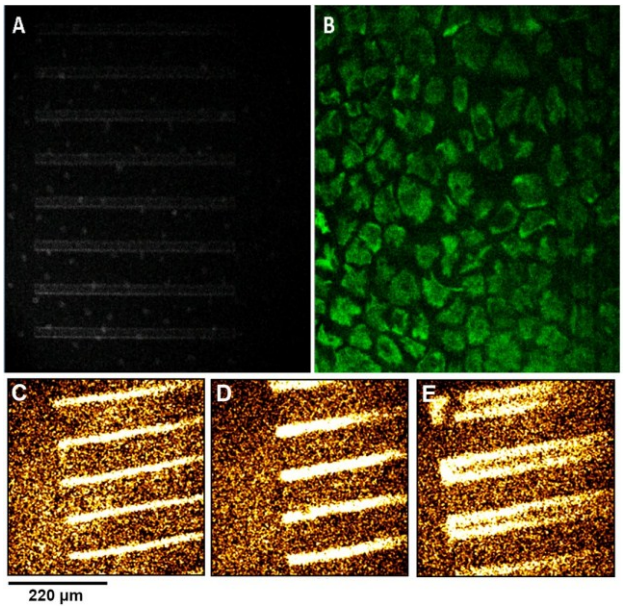

Figure 2. *Upper* Non-patterning of astrocytes for UV ablated parylene-C. A) highlights grey parylene-C strips of 20µm and nuclei coverages are grey circular regions, B) highlights the random astrocyte coverages. *Lower* O 1s (537.1eV) XPS images of 20, 40 and 100µm parylene-C strips (C), D) and E) respectively), highlighting oxidized carbon. Larger strip widths we used in (c-e) to highlight the effect of how the photo-oxidation penetrated the material with strip-width.

The extent of photo-oxidation of UV laser ablated parylene-C/SiO_2 was investigated using X-Ray Photoelectron Spectroscopy (XPS) for strip-widths of 20, 40 and 100µm at

a pulse fluence of 250mJcm^{-2}. The larger strip widths shown in Figure 2(c-e) where used to highlight the effect of how the photo-oxidation penetrated the material with strip-width. The presence of oxygen, indicated by the O 1s orbital, is indicative of carboxyl groups at the surface of the parylene-C caused by photo-oxidation [12], [17], [18]. For the 20 and 40µm wide strips oxidation was observed over the entirety of the parylene-C surface whereas for the 100µm wide strip, in Figure 2(e), oxidation was observed at the edges of the strip but not the centre. This could be due to diffraction of the laser beam outside the mask, i.e. the diffracted light may be sufficient to cause photo-oxidation but not intense enough to cause ablation.

Thus, it is evident that coherent UV can cause photo-oxidation of the adjacent parylene-C strips sufficiently to disrupt cell patterning.

B. IR Laser Ablative Patterning

It was found that a single pulse at the highest fluence available, 684mJcm^{-2}, was sufficient to completely ablate the parylene-C without significant damage to the underlying substrate.

The best cell patterning was found to occur on the 20µm strip widths for both multiple pulse low power and single pulse high power ablation. However, for the multiple pulse low power IR ablation, our recent study in [19] revealed that ragged veneers where also present around the edges of the parylene-C strips as opposed to the straight edges that occurred with the single pulse high power ablation. Hence, we elected to use the single pulse high power due to optimize the high resolution of the edges of the parylene-C strips. For single pulse high power ablation the 20um wide strips provided a 28% cellular coverage and 12% nuclear coverage and a 5.6:1 contrast between the cells on the parylene-C and those on the SiO_2 substrate. These results are comparable to our previously published hNT astrocyte patterning on photolithographic produced parylene-C/SiO2 substrates [8] which provided an astrocyte cell process coverage of 40% with 12% nuclear coverage for the same parylene-C strip dimensions.

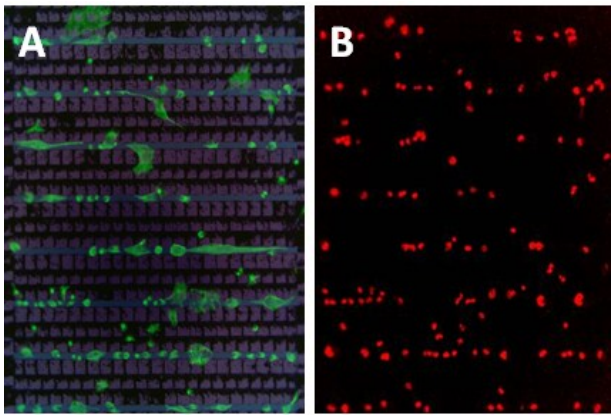

Figure 3. Patterning of hNT A) Astrocytic processes and B) Nuclei on parylene-C/SiO_2 substrates using single pulse high power ablation.

Cell patterning occurred along the parylene-C strips as expected from [5-8] due to the surface chemistry between adsorbed fetal bovine serum (FBS) and the parylene-C. However, it should be noted that to avoid a gap between the 50x50µm square ablations the pulse overlap of the laser was set to the smallest distance available of 5µm. This, tradeoff in unavoidable overlap (highlighted as the black regions in Figure 3(a)) was found to create nano-scale roughness which served to attract neuronal cell growth to these regions. Hence, it can be seen that a small amount of growth is evident in such regions on the chip, as highlighted in Figure 3(a). The topography of the parylene-C also influenced the direction of the patterning by forcing the star-like astrocytes conform to the thin narrow strips prescribed. For our work which is concerned with activating cells at the single cell level, such thin strips are essential in obtaining single cell resolution which was achieved and as is evident in Figure 3(b).

IV. Conclusion

In conclusion, both UV and IR laser ablation were effective in micro machining the patterned parylene-C strips. However, it was found that hNT astrocytes did not pattern on UV ablated substrates, due to photo-oxidation under UV light. IR laser ablation in contrast provided high yield patterning comparable to standard photolithography methods. While the cellular process coverage was reduced in comparison to standard photolithographic work, the nuclear coverage was preserved and single cell isolation was observed. Thus, IR laser ablated parylene-C/SiO_2 chips offer a good yield, low cost alternative to standard photolithographic techniques for patterning cells on chip. Thus, by making a samll comprise of the astrocytic cell coverage it is possible to avoid potentially expensive photo-mask and difficult photolithographic processing steps. Furthermore, this technique allows a user to realize many different chip designs, without incurring expensive photo-mask redevelopment costs. This promises to reduce costs and simplify research in the areas of cellular micro-arrays, microfluidics and multi-electrode array applications.

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