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First Human hNT Astrocytes Patterned to Single Cell Resolution on Parylene-C/Silicon Dioxide Substrates

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Abstract— **In our previous work we developed a successful protocol to pattern the human hNT neuron (derived from the human teratocarcinoma cell line (hNT)) on parylene-C/SiO_2 substrates. This communication, reports how we have successfully managed to pattern the supportive cell to the neuron, the hNT astrocyte, on such substrates. Here we disseminate the nanofabrication, cell differentiation and cell culturing protocols necessary to successfully pattern the first human hNT astrocytes to single cell resolution on parylene-C/SiO_2 substrates. This is performed for varying parylene strip widths providing excellent contrast to the SiO_2 substrate and elegant single cell isolation at 10µm strip widths. The breakthrough in patterning human cells on a silicon chip has widespread implications and is valuable as a platform technology as it enables a detailed study of the human brain at the cellular and network level.**

I. Introduction

A fundamental question that still remains in the field of neuroscience is; "How do the cells of a neural circuit and their connectivity to one another contribute to large scale network behaviour as a whole ?". The reason this question remains unanswered is due to the fact that the neurons and glial cells of the brain grow in a complex interwoven fashion. Thus, it is difficult to control the morphology to which cultured neurons and glia grow and the connections that they make to one another in order to study them effectively. In neurophysiology, the "patch clamp" [1] is generally used to investigate groups of connected cells. However, patch clamp work confines the study to only 2-3 neurons due to the physical limitations of the patch clamp experiments. In addition, the removal of the micropipettes necessary in patch clamp studies limits the life-time of the cell [2] thus hindering the chance of repeating the experiment.

The field of 'Cell Patterning'[3] endeavors to control the precise placement and arrangement of cells into organised networks such that simple network architectures can be investigated in a controlled manner. Cell patterning employs a variety of techniques, mainly using silicon chip technology, such as micro-contact stamping [4], immobilisation [5], micro-fluidics [6] and photo-lithographic techniques [7] to control the connectivity problem by allowing ensembles of neurons and glia to be patterned on silicon.

Silicon chip technology is generally employed in cell patterning as 'Multi-Electrode Arrays (MEAs)' [2,8] can be further embedded in the semiconductor substrate in order to investigate the signaling that exists between cells. This combination of technologies promises key advancements in neuroscience [3].

Typical, brain cell studies on chip usually employ cells from small model organisms such as the embryonic rat. However, to better model the pathological human brain, it is preferential to utilise human brain cells derived from human tissue as their properties better match the human cell types than the embryonic rat cell types [9]. Human cells can be obtained in a variety of ways: Umbilical cord; embryonic tissue; postmortem tissue; cancerous tissue and from stem cell lines

Human embryonic neural progenitor cells have been patterned on chip [10], however, they are not widely available as are cells from the umbilical cord. In addition, post-mortem cells [11] are harder still to acquire and have not been patterned on chip. Thus, stem cell lines provide an accessible way to provide large quantities of well characterised human neurons and astrocytes [12].

In our work we have elected to use the human hNT astrocyte (derived from the human teratocarcinoma cell line (hNT)). The human hNT cell line [13] was chosen as it expresses ubiquitous neuronal and astrocytic markers [15], is widely available and provides the closest model to adult human neural and astrocytic tissue [15]. Furthermore, this human stem cell line raises no ethical concerns as the astrocytes were differentiated from a cancer stem cell line rather than an embryonic brain tissue[13].

In our work, we also employ a patented Parylene-C/SiO_2 interface [16] which has been demonstrated to be a reliable and robust photolithographic technology for cell patterning of rat neurons and glia [17,18]. Parylene-C (poly-monochoro-para-xylylene), known as DiX C commercially, is a derivative of Parylene which is the generic name of the class of poly-p-xylylene polymers deposited by chemical

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vapor deposition (CVD) [19]. Parylene-C differs chemically from other derivatives of its chemical class having one chlorine atom per monomer benzene ring. This molecular arrangement provides properties such as: low moisture and gas permeability; fast deposition; a uniform conformal surface; pinhole free deposition; good electrical insulation and resistance to chemical attack. Parylene-C is a biocompatible material employed in permanent medical implants such as stents, pacemakers and defibrillators as a moisture barrier [20].

Cell guidance with parylene-C has been demonstrated in stencils, electrically modifiable hydrophobic/hydrophilic bases and in 3D cages [21,22]. Furthermore, parylene-C's cell adhesive capabilities have been examined under certain conditions [23,24]. The authors have demonstrated that it is possible to accurately pattern primary neurons and astrocytes [17,18] from the embryonic rat. More, recently we have developed a protocol to pattern the human hNT neuron [25] on parylene-C/SiO_2 interfaces in a reliable and robust manner.

II. Motivation

There are approximately 100 billion neurons in the human brain with the quantity of astrocytes existing at an order of magnitude larger than the neuronal content. The astrocyte has been quite an elusive cell effectively evading the definition it was originally given [26].

Originally, it was commonly considered a supportive cell to the neuron by physically supporting them spatially in the brain and supplying the neurons with blood from neighbouring blood capillaries. Recently, however, new functionality has been gleaned from this elusive cell [26].

Our motivation for patterning the astrocyte is to help facilitate single cell and network studies into the undiscovered functionality of this interesting cell.

The objective to this work is to achieve two aims: to determine if it is possible to pattern the hNT astrocyte on a parylene-C/SiO_2; and to determine what strip widths of parylene-C provides single-cell isolation and so enable future single cell and network studies in this area.

III. Methods

A. Micro-fabrication of Parylene-C on SiO_2

Silicon wafers were passivated in a furnace (H_2 1.88sccm and O_2 1.25sccm) for 40 minutes at 950^{o}C producing a 200nm SiO_2 layer measured with a Nanometrics NanoSpec/AFT, Microarea gauge. From [17] 100nm of Parylene-C was deposited on the passivated wafers at a rate of 1.298 nm per mg of dimer using a Labcoter 2 deposition Unit (Model PDS2010) at room temperature. Hexamethyldisilazane (HMDS) was then deposited on the parylene coated wafers in an SVG 3 inch photo-resist track. Positive photo-resist of 1mm was then deposited on the wafers with a Rohm & Hass SPR350-1.2 (spin speed of 4000 rpm for 30 seconds) followed by a 60s soft bake at 90^{0}C. Both the wafers and photo mask were placed in an Optimetrix 8605 5x reduction stepper to produce parylene printed strips of strip length 500μm and strip widths of 5, 10 and 20μm. The separation between strips was 120μm (~10 cell body diameters to avoid cell cross-over between adjacent strips).

A 60s bake at 110^{0}C was performed, removing exposed photo-resist from the wafers after development in Microchem MF-26A developer. The wafers were placed in a Plasmatherm for 90s (at a 50 mTorr chamber pressure, 50 sccm O_2, 500W RF power) which etched off unwanted parylene (at an etch rate of ~100 nm/min) to reveal the SiO_2 substrate. (The SiO_2 on the etched areas was validated by Nanospec measurement). Residual photo-resist was removed by spinning acetone on the wafers. The wafers were cut with a DISCO DAD 800 Dicing Saw (speed 30 000 rpm, feed speed 7 mm/s). The wafers were then rinsed in distilled ionized water and blown dry with nitrogen.

B. Chip cleaning

Piranha acid (5:3 ratio of 30% Hydrogen Peroxide (H_2O_2) and 98% Sulphuric Acid (H_2SO_4)) was used to clean the chips for 10 min in a clean room. The chips were then rinsed 3 times in distilled water and blown dry with nitrogen.

C. Stem Cell Differentiation into hNT Astrocytes

The hNT astrocytes were produced from a NTera2/D1 cell line, purchased from the American Tissue Culture Collection (ATCC; CRL-1973). All reagents that follow, in parts C-E, were purchased from Invitrogen unless stated otherwise. Cells were cultured in DF10 (DMEM/F12 media with 10% foetal bovine serum (FBS) and 1% Penicillin-Streptomycin-Glutamine). Cells were split 1:5 when they reached 100% confluence. This was performed 2-3 times a week and the media was changed every second day. The NTera2/D1 cells are a precursor cell line with the potential to differentiate into mixed neuronal cultures (referred to as hNT neurons and hNT astrocytes) [13,14]. Following a protocol developed in [27] with modifications developed now described. This protocol enables the hNT neurons to be harvested after 7 weeks of differentiation and the hNT astrocytes 4 weeks later following further mitotic inhibition.

Precursor NT2 cells were seeded at ~2.5 million cells per T75 culture flask to settle for 24 hours. The media was replaced with DF10 supplemented with 10μM retinoic acid (RA). The media was refreshed every second day and the cells were allowed to differentiate in the presence of RA for 4 weeks. After the differentiation period, the cells were trypsinised and split 1:2 and cultured in DF10 for 3 days. After this period non-adherent cells were discarded, the remaining adherent cells were trypsinised, counted and seeded at 25-30 million cells per T75 flask. After a 3 day recovery period in DF10 the media was harvested to use as conditioned media (CM). The CM was diluted 1:1 with DF10 and supplemented with 3x mitotic inhibitors (MI; 10 μM cytosine arabinoside, 10 μM fluorodeoxyuridine (FUrd) and 10 μM uridine (Urd) (all from Sigma). The partially differentiated cells were then grown in the 3x MI cocktail for 1 week [25,27]. At this stage of the differentiation process, neurons were harvested as described in [25]. Remaining cells were then cultured for a further 2 weeks in

the presence of 10 μM FUrd and 10 μM Urd. Finally, cells were cultured for a further 2 weeks in 1x MI containing 10 μM Urd. The 2x and 1x MI cocktails were replenished every three days. All of the mitotic inhibition was conducted using DMEM/F12 containing 5% FBS.

After the 10 week differentiation protocol, astrocytes were harvested with a 3-4 minute trypsinisation (0.5% solution) followed by brisk striking of the flasks to dislodge the astrocytic cells. The astrocytes were rinsed with DF10 media and centrifuged at 200 xg, counted prior to use, and assessed to ensure the astrocytes were single cell suspensions.

D. Seeding of Cells onto Chip

The chips were treated with 1% pen Strep solution for 1 hour and then rinsed gently with sterile water. All chips were then immersed in foetal bovine serum (FBS) with a serum immersion time of 3 hrs. The same water immersion times were applied to a control group. All were incubated over this period in order to activate the parylene-C strip patterns.

After the immersion times were completed, excess serum was gently rinsed off and the hNT astrocyte cells were seeded onto the chips in DF10 media. A seeding density of ~100 cells/mm^2 was found to provide the best. The cells were then allowed to migrate and adhere to the parylene strips. Media was changed after 3 days with fresh DF10, the cells were grown for a further 3 days and fixed with 4% paraformaldehyde (PFA) for 10 minutes, followed by rinsing and permeabilisation of the cells with PBS containing 0.2% triton-X100.

E. Labelling & Imaging the Cells

The permeabilised astrocytes were stained using an anti-vimentin antibody (abcam; ab15248). Primary staining was conducted at 1:1000 and detected using anti-rabbit Alexa 488 (Invitrogen; 1:400). Staining for the intermediate filament vimentin provided considerably better cellular resolution than live cell stains such as CMFDA or CFSE. Vimentin also provided the best labelling to clearly identify the cell bodies and the surrounding cytoplasm separately. Nuclei were stained using Hoechst 33258. The chips were mounted using AF1 (a 50:50 Phosphate buffered saline) PBS:glycerol mix (Citifluor).

The images obtained are shown in Fig. 1, were taken using a Leica DM IRB microscope coupled with a Leica DC 100 digital camera. Images were acquired using a 20x objective lens.

IV. RESULTS

The 3 images in Fig. 1 are for 500μm strip lengths of parylene-C deposited on SiO_2 for parylene-C strip widths of 20μm, 10μm and 5μm. Fig. 1A, depicts the 20μm parylene-C strip widths. Good conformity to the parylene–C was obtained with the hNT astrocytes with successful migration to the parylene-C strips. A relatively good contrast was obtained. However, small amounts of the astrocytic processes are observed extending from some of parylene-C strips to others. Single cell isolation at 20μm was observed but mainly 2-3 cells existed in small clumps at this width.

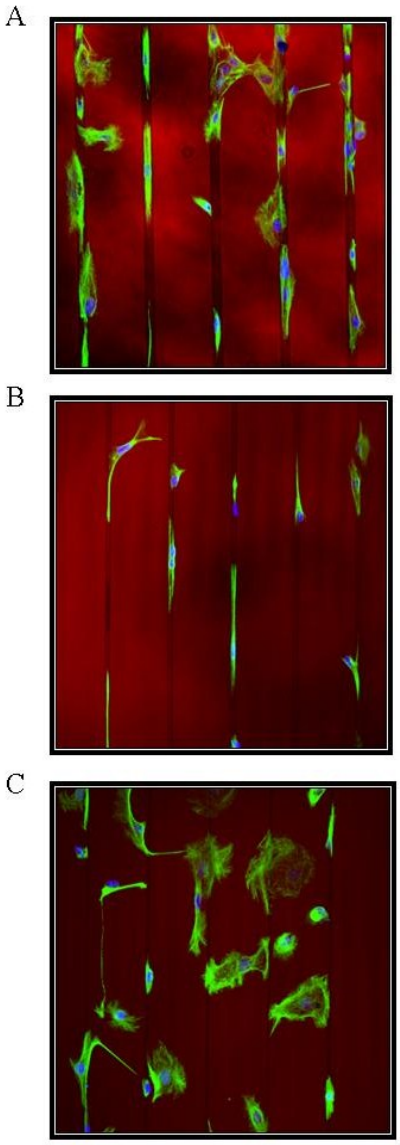

Fig. 1. The first hNT astrocytes patterned on parylene-C/SiO_2 substrates at 500μm strip lengths: A. 20μm; B. 10μm and C. 5μm parylene-C strip widths. Vimentin stained astrocytes are green and nuclei are blue (Hoechst 33258). Single cell isolation is clearly evident and an excellent contrast to the SiO_2 is demonstrated at 10μm strip widths.

Fig. 1B, depicts the 10μm parylene-C strip widths. Excellent conformity to the parylene–C and contrast to the SiO_2 was clearly observed. Mainly, the whole astrocyte cell body and its dendritic processes were found to conform along the parylene-C strips spanning distances of up to 150μm. In rare case, astrocytes dendritic processes were observed both on and off parylene-C strips. However, the authors believe that this is astrocytes moving onto the parylene-C from the SiO_2 rather than off. Single cell isolation at 10μm was observed to be excellent. However, in a few uncommon instances aggregates of 2 cells occurred. A detailed study of the patterning of hNT astrocytes has recently been accepted in [28] which addresses the technical details, statistical analyses and repeatability of such work.

Fig. 1C, depicts the 5µm parylene-C strip widths. Poor conformity to the parylene–C and contrast to the SiO_2 was found at this strip width resolution. It was found that the whole astrocyte cell body and its dendritic processes mainly occurred on the SiO_2 rather than the parylene-C strips and resembled the typical star-like morphology for these astrocytes evident in our water controls. The physiological effects of such elongation are yet to be determined for future studies.

V. Conclusion

In conclusion, this work has successfully demonstrated the patterning of the first human hNT astrocyte on parylene-C/SiO_2 substrates, 100nm thick. In addition, it was identified that a parylene-C strip width of 10µm provided excellent conformity to the parylene-C with the astrocytes dendritic processes spanning distances of up to 150µm. Furthermore, excellent single cell isolation was obtained at the 10µm strip width. The findings presented here demonstrate successful conformity, contrast and single-cell isolation for hNT astrocytes on parylene-C/SiO_2. This will open up the possibility to exploit parylene-C as an alternative biomaterial in cell patterning and pave the way in the facilitation of future single cell and network studies of the human hNT astrocyte.

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