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Short communication

First human hNT neurons patterned on parylene-C/silicon dioxide substrates: Combining an accessible cell line and robust patterning technology for the study of the pathological adult human brain

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ABSTRACT

In this communication, we describe a new method which has enabled the first patterning of human neurons (derived from the human teratocarcinoma cell line (hNT)) on parylene-C/silicon dioxide substrates. We reveal the details of the nanofabrication processes, cell differentiation and culturing protocols necessary to successfully pattern hNT neurons which are each key aspects of this new method. The benefits in patterning human neurons on silicon chip using an accessible cell line and robust patterning technology are of widespread value. Thus, using a combined technology such as this will facilitate the detailed study of the pathological human brain at both the single cell and network level.

1. Introduction

A significant gap exists in our current understanding of how individual cells in the brain connect to form and drive large scale network behaviour. This question remains unanswered due to the highly complex and entwined growth of neuron and glial cells in the brain. Random cultures of neurons and glia offer little control over the morphology, synaptic connections and type of individual cells, to facilitate an effective network study. The precise placement and organization of individual neurons and synapses promises key advancements in neuroscience as interactions can be studied both at the cellular and network level (Wheeler and Brewer, 2010).

Furthermore, brain cell studies usually employ neurons from small model organisms such as the embryonic rat for reasons such as well-known anatomy, low cost and quick reproduction. However, if our goal is to contribute to studies of the pathological human brain, it is preferential to utilise neurons derived from human neuronal cell lines, as their properties better match the adult human neuron than the commonly used embryonic rat neuron (Howard et al., 2008). Whilst embryonic neural progenitor cells have recently been patterned on chip (Regan, 2010) they have the disadvantage that they are not widely available. Post-mortem neurons (Palmer et al., 2001) are harder still to acquire and have not been patterned on chip. Thus, cell lines are the most accessible way to provide large quantities of well characterised human neurons (Svendsen et al., 1998).

This communication reports how we have recently succeeded in patterning the first human hNT neurons on parylene-C/silicon dioxide substrates. The human hNT neuron (Andrews, 1984) was chosen as it expresses ubiquitous neuronal markers (Pleasure et al., 1992), provides the closest model to adult human neural tissue (Görtz et al., 2004) and is widely available. The parylene-C/silicon interface has been recently pioneered and demonstrated to be a reliable and robust photo-lithographic technology for cell patterning of rat neurons and glia (Murray, 2008; Delivopoulos et al., 2009). This brief communication describes the detailed cell culturing and photo-lithographic methods that were developed to achieve successful patterning of the human hNT neurons on chip.

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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. This could eventually lead to new drug treatments for a range of adult brain pathologies, such as Epilepsy, Alzheimer's and Parkinson's disease. This study raises no ethical concerns as the neurons were differentiated from a cancer (Andrews, 1984) stem cell line rather than an embryonic brain tissue.

2. Materials and methods

2.1. Micro-fabrication of parylene-C strips on SiO_2 substrates

Initially, silicon wafers were passivated in a furnace (H_2 1.88 sccm and O_2 1.25 sccm) at 950 °C for 40 min to produce a 200 nm SiO_2 layer (measured with a Nanometrics NanoSpec/AFT, Microarea gauge). 100 nm of the biomaterial, parylene-C (Delivopoulos et al., 2009), was then deposited at room temperature on the passivated wafers at a rate of 1.298 nm per mg of dimer using a Labcoter 2 deposition Unit (Model PDS2010). Hexamethyldisilazane (HMDS) was then deposited on the parylene coated wafers in an SVG 3 in. photo-resist track. 1 mm of positive photo-resist was then applied to the wafers using a Rohm & Hass SPR350-1.2 at a spin speed of 4000 rpm for 30 s. This was followed by a 60 s softbake at 90 °C. Both the wafers and photo-mask were placed in an Optimetrix 8605 5× reduction stepper to produce parylene printed strips of strip length 1000 μm and strip width of 30 μm. The separation between strips was 120 μm (~10 cell body diameters).

A 60 s bake at 110 °C was then performed and exposed photo-resist was removed from the wafers after developing in Microchem MF-26A developer. Insertion into a Plasmatherm for 90 s (at a 50 mTorr chamber pressure, 50 sccm O_2, 500W RF power) etched off unwanted parylene, not protected by the photo-resist (at an etch rate of ~100 nm/min) to reveal the SiO_2 underneath. (The SiO_2 on the etched areas was validated using the Nanospec). Any residual photo-resist was removed by spinning acetone on the wafers on the photo-resist track. Finally, the wafers were cut with a DISCO DAD 800 Dicing Saw (spindle speed 30,000 rpm, feed speed 7 mm/s). The wafers were then rinsed in distilled ionized water and blown dry with nitrogen.

2.2. Chip cleaning

The chips were cleaned with piranha acid (5:3 ratio of 30% hydrogen peroxide (H_2O_2) and 98% sulphuric acid (H_2SO_4)) for 10 min in a clean room. The chips were then rinsed 3 times in distilled water and blown dry with nitrogen. They were then transferred to dust free cases.

2.3. Differentiation of the NTera2/D1 cell line into neurons (hNT neurons)

The NTera2/D1 cell line was purchased from the American Tissue Culture Collection (ATCC). Cells were cultured in DF10 (DMEM/F12 media supplemented with 10% foetal bovine serum (FBS) and 1% penicillin–streptomycin–glutamine). Cells were split 1:5 when they reached 100% confluence, 2–3 times a week and media was changed every second day. All reagents were purchased from Invitrogen (unless otherwise stated). The NTera2/D1 cells are a precursor cell line with the potential to differentiate into mixed neuronal cultures (referred to as hNT neurons) and astrocytes (Andrews, 1984; Pleasure et al., 1992). We followed a protocol developed in Lim et al. (2007) with modifications now described. This protocol enables neurons to be harvested after 7 weeks of differentiation.

Typically, precursor NT2 cells were seeded at ~2.5 million cells per T75 culture flask to settle for 24 h. The media was then replaced with DF10 supplemented with 10 μM retinoic acid (RA). This media is refreshed every second day and the cells differentiated in the presence of RA for 4 weeks. Following this differentiation period, the cells are trypsinised and split 1:2 and cultured in DF10 for 3 days. After this period non-adherent cells are discarded, the remaining adherent cells are trypsinised, counted and seeded at 25–30 million cells per T75 flask. Following a 3 day recovery period in DF10 the media is harvested to use as conditioned media (CM). The CM is diluted 1:1 with DF10 and supplemented with 3× mitotic inhibitors (MI; 1 μM cytosine arabinoside, 10 μM fluorodeoxyuridine and 10 μM uridine (all from Sigma). The partially differentiated cells were then grown in the 3× MI cocktail for one week (Lim et al., 2007). It should be noted that during this period a neuronal phenotype is evident and these cells can easily be dislodged. Thus, care is required when handling the cultures. The neurons were harvested with a 1 min trypsinisation followed by brisk striking of the flasks, which dislodged the neuronal cells. The neurons are centrifuged at 200 × *g*, counted prior to use, and maintained in DF10 media for several days until required for seeding onto the chips.

2.4. Seeding of cells onto chip

The chips were treated with 1% pen Strep solution (Invitrogen) for 1 h and then rinsed gently with sterile water. The chips were divided into 4 groups. Three of the groups were then immersed in one of 3 sera and the fourth group in a water control. The sera used were equine, AB-Human Serum and foetal bovine serum (FBS) all purchased from Invitrogen. One half of each group of chips had a serum immersion time of 3 h whilst the other half had an immersion time of 24 h. The same water immersion times were applied to the control group. All were incubated over this period in order to activate the parylene strip patterns. After the immersion times were completed, excess serum was gently rinsed off and the neuronal cells were seeded onto the chips in DF10 media. After some initial optimisation a seeding density of ~100 cells/mm^2 was determined to provide the best results. The cells were allowed to adhere with the media being changed after three days with fresh DF10, the cells were grown for a further 3 days and fixed with 4% paraformaldehyde (PFA) for 10 min.

2.5. Labelling and imaging the hNT neurons

It was found that CMFDA (5-chloromethylfluorescein diacetate) commonly used for live-labelling of cells provided the best labelling for the neuron culture for both intensity and the fact that it stains the entire neuronal cytoplasm. The chips were mounted between using AF1 (a 50:50 (phosphate buffered saline) PBS:glycerol mix purchased from Citifluor. The images obtained, shown in Fig. 1, were taken using a Leica DM IRB microscope coupled with a Leica DC 100 digital camera. The objective lenses used were of magnifications ×10 and ×20.

3. Results (see attached figures + attached figure captions)

3.1. Effect of serum type and serum immersion time

Fig. 1, highlights how well the hNT neurons migrated and conformed to the parylene surfaces for 3 different serum types (equine, human and FBS) and 2 different serum immersion times (3 h and 24 h). Fig. 1(E) demonstrates how immersion of the chips in FBS for 3 h produced strong distinct lines of hNT neurons that conformed to the parylene strip patterns. This was found to be superior to the chips that were immersed in equine and human serums for

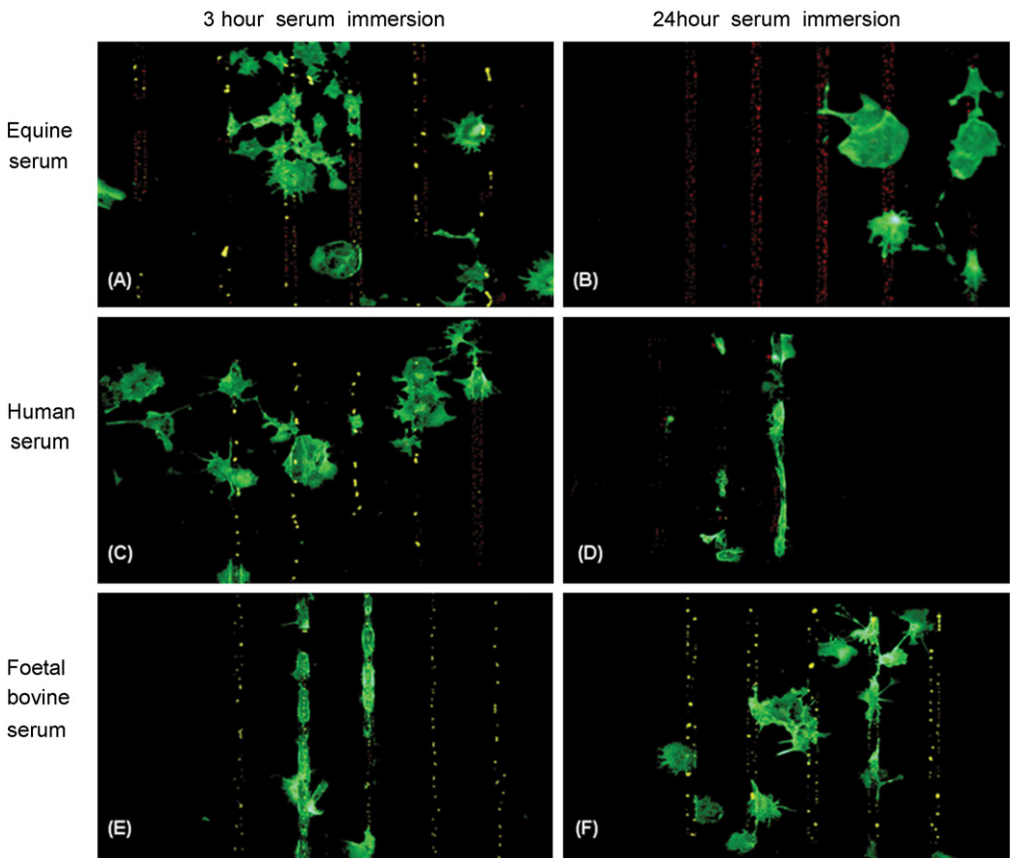

Fig. 1. Human teratocarcinoma cell line-derived (hNT) neurons cultured on parylene-C/silicon dioxide substrates for different serums and serum immersion times. All parylene strips were 1 mm in length, 30 μm in width, 100 nm thickness, at 120 μm spacing, plated at 100 cells/mm^2, taken at 4.2 s exposure time at ×10 magnification; (A) equine serum 3 h immersion time; (B) equine serum 24 h immersion time; (C) human serum 3 h immersion time; (D) human serum 24 h immersion time; (E) FBS 3 h immersion time; (F) FBS 24 h immersion time. Auto fluorescence is exhibited as red and yellow stippling in the images (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.).

the same period of time Fig. 1(A and C respectively). In addition, immersion of the chips in FBS for 3 h was also superior to immersion in FBS, equine and human serums for 24 h, Fig. 1(B, D and F respectively). However, immersion of chips in human serum for 24 h, Fig. 1(D), did produce a higher level of conformity to the parylene than equine serum (3 h and 24 h) and FBS serum (24 h) but was less than immersion in FBS for 3 h (discussed further in Section 4). The 3 h immersion time in FBS produced a very sharp cellular contrast on the parylene as opposed to the SiO_2 substrate whereas other serum types and immersion times produced a blotchy patterning of the neurons which spanned across the parylene and onto the SiO_2 substrate.

In order to achieve a better image contrast, a longer microscope exposure time was used for the superior FBS 3 h sample determined from Fig. 1. Fig. 2(A) highlights parylene strip lengths of 1000 μm immersed in FBS for 3 h immersion time. Fig. 2(B) highlights a brief trial on smaller strip lengths of 250 μm which demonstrates the same level of conformity as the 1000 μm strip lengths, of Fig. 2(A). Fig. 2(C) is a 24 h water control at magnification ×20 which produced a blotchy patterning on the parylene. However, off the parylene (i.e. on the SiO_2) the water control demonstrated typical cell morphology of hNT neurons where projections are sent out in all directions.

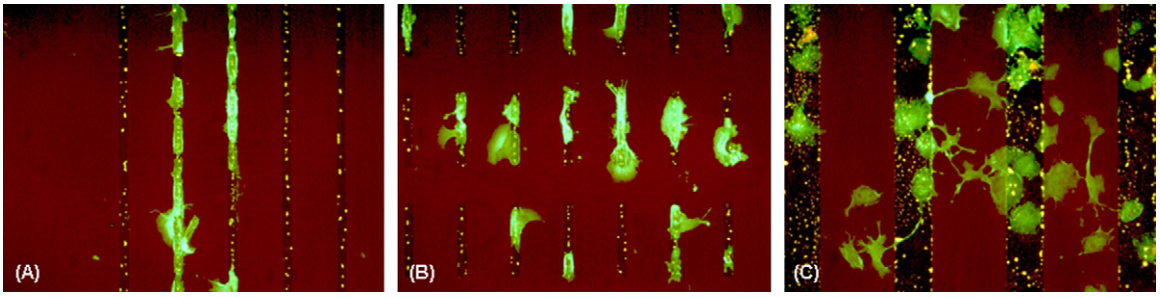

Fig. 2. Human teratocarcinoma cell line-derived (hNT) neurons cultured on parylene-C/silicon dioxide substrates at 7 s exposures for large and small strip lengths and a water control to highlight the cell morphology. All parylene strips were 30 μm in width, 100 nm thickness, at 120 μm spacing, plated at 100 cells/mm^2, taken at ×10 magnification (unless otherwise stated); (A) FBS 3 h immersion time, 1000 μm strip lengths; (B) FBS 3 h immersion time, 250 μm strip lengths; (C) water control 24 h immersion time ×20 magnification. Auto fluorescence is exhibited as red and yellow stippling in the images (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.).

4. Discussion

In this communication, we describe the detailed nanofabrication processes, cell differentiation and culturing protocols necessary to successfully pattern hNT neurons onto parylene-C/silicon dioxide substrates. Although not documented, it is well-known in the field of hNT cell growth that it is difficult to get hNT neurons to adhere to surfaces in general. Thus, the work performed here offers a viable method to produce adherence of hNT neurons to parylene-C for further study of this cell type.

It was elected to perform the initial work on the same 100 nm parylene thickness, as reported in the original work (Delivopoulos et al., 2009) that cultured primary rat neurons on a parylene-C/silicon dioxide substrate. Here we opted to use parylene strips of 30 μm in width as the hNT neuron cell bodies are slightly larger than the primary rat neuronal cells which preferred 20 μm (Delivopoulos et al., 2009). The hNT protocol, developed here, established that 100 cells/mm^2 and that 3 h serum immersion of the chips using FBS was optimal for successful culture of the hNT neurons. In contrast, the rat neurons preferred equine serum and the best growth conditions were obtained using a lower seeding density of 70 cells/mm^2. These are subtle but important differences required for the successful culture of hNT neurons. We hypothesise that this is due to the different receptors in the hNT and primary rat neurons that probably interact with different proteins in foetal bovine and equine serums respectively.

Although it was found that immersion in human serum for 24 h gave a good level of conformity (which was only secondary to a 3 h immersion in FBS), FBS is favoured as a lower cost option to that of human serum (as human serum is approximately 10 times the cost of FBS) unless the human serum significantly outperformed the FBS results. It was also found that the hNT neurons grew best using a 6 day culturing time on chip which was shorter to that of the primary rat neurons.

It was shown how the hNT neurons successfully migrated to parylene-C lines 1 mm in length, 30 μm in width, 100 nm thickness and at a 120 μm spacing. It should be noted that the hNT neurons are approximately 20–30 μm in diameter and do not divide after differentiation (Andrews, 1984) because of the period spent differentiating in mitotic inhibitors (discussed in Section 2.3). Thus, the number of hNT neurons deposited on chip remains constant. Due to this fact, the figure demonstrates large network formation spanning distances of up to approximately 1 mm (~100 cell bodies in series). We would like to emphasise that the unusual longitudinal shape of the neurons is caused by their preferential adhesion to the parylene surface thus conformity to the longitudinal geometry of the parylene strips. In addition, it was shown that the same level of conformity was achieved for smaller parylene strip lengths of 250 μm.

The work presented in this brief communication, demonstrates for the first time that human neuronal cells derived from Ntera/D1 stem cells can be successfully patterned on the biomaterial parylene-C. Our optimised culture conditions produced excellent contrasting selectivity for adhesion of the neurons between the parylene and silicon dioxide substrate. This protocol is represents a foundation for future studies investigation neuronal connectivity and communication employing this new technology. The patterning of adult human neurons on chip provides the important first steps to enable a detailed study of the signalling in adult human brain circuits at the single cell and network level for clinically significant human brain pathologies such as epilepsy, Parkinson's and Alzheimer's disease which affect millions of people each year.

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