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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