Diamond & Related Materials 23 (2012) 100–104

Contents lists available at SciVerse ScienceDirect

Diamond & Related Materials

journal homepage: www.elsevier.com/locate/diamond

Spatially controlling neuronal adhesion on CVD diamond

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ARTICLE INFO

Article history:
Received 7 November 2011
Received in revised form 20 January 2012
Accepted 23 January 2012
Available online 28 January 2012

Keywords:
Diamond
Cell adhesion
Neural cell
Patterning

ABSTRACT

The mechanical and chemical properties of CVD diamond films make them very suitable materials for improving the long-term performance of invasive electrode systems used in brain-computer interfaces (BCIs). We have performed *in vitro* testing to demonstrate methods for spatially directing neural cell growth and limiting the detrimental attachment of cells involved in the foreign-body response on boron-doped diamond. Laser micro-machining techniques were used to control neuronal adhesion and to modify inflammatory-cell attachment. Laser micromachining was used to etch 100 μm × 1000 μm patterns into poly-D-lysine-coated B-doped diamond. Rat cortical neurons grew well across the coated areas and neuritic outgrowth largely avoided crossing over the etched areas. This avoidance made the etched pattern visible upon staining of the cells and their processes.

This work demonstrates that patterns etched into diamond form a spatially defined substrate for organizing neural cell growth. Although preliminary, this work could have further applications in neural-network research and nerve and brain repair scaffolds.

1. Introduction

Diamond is chemically and biochemically inert, by which we mean that biological cells in contact with diamond surfaces show little or no cytotoxic response [1,2]. This makes diamond an ideal material for coating medical implants [3,4], artificial retinas [5,6], or for use as a substrate upon which to grow biological cells [6-10]. The findings show that for nearly all the cell types (neurons, osteoblasts, fibroblasts and standard cell lines) studied in these reports *no* deleterious or cytotoxic responses were observed on diamond, and often there was slight improvement in cell proliferation over standard glass substrates. Recent studies at the National University of Singapore [7] and at Kassel University in Germany [11] show that oxygen termination of the diamond surface improved the cell adhesion, since the surface carboxyl groups appeared to mimic those on proteins and biomolecules. Improved human osteoblast proliferation and the stimulation of differentiated markers have been observed on a nanocrystalline diamond surface, which may be useful for bone regeneration purposes [8,9]. In addition, diamond films have been shown to exhibit the highest resistance to bacterial colonization when compared to medical steel and titanium [12], a relevant issue since bacterial infection associated with the use of biomaterials is still a significant clinical problem.

Being able to culture, modify, control and make electrical connections to neurons opens up the possibility of direct brain-computer interfaces (BCI). The attractive possibility of being able to restore function to sufferers of spinal injury, degenerative disorders, such as cerebrovascular disease, brain-stem stroke, cerebral palsy and many other neuromuscular disorders, drives the need for advancing neural-interface technologies [13-15]. Despite such advances in intra-cortical implants, electrode systems implanted into mammalian central nervous systems often fail as a result of an unwanted foreign-body response [16]. Analyses of the failure showed that the electrodes had often become encapsulated by *glia* cells (non-neuronal cells that maintain homeostatis and provide support and protection for neurons) in a process known as *glial scarring*. Improving neuron-electrode connectivity by reducing glial activity and preventing the subsequent scarring has therefore become an important area of BCI research.

To reduce glial scarring, a number of groups have begun to consider diamond and DLC for the base material for neural-implant coatings [11,17-23]. The chemical inertness, wear resistance and bioinertness of these materials provide protection from the harsh enzymatic and degradative environment of the body. Moreover, both materials can be modified with dopants to alter their mechanical, chemical, electrical and biological properties.

In 2004, pioneering work done at University College London showed that mice cortical cells could be successfully grown on single-crystal diamond surfaces [24]. The attached neurons developed long neurites during the first 24 h of culturing, and formed more complex neurite patterns within 48 h. Last year the same group showed [25]

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0925-9635/$ – see front matter
doi:10.1016/j.diamond.2012.01.023

that neurons cultured on various nanodiamond-coated substrates perform similarly to those grown on standard protein-coated materials with respect to their initial cell attachment, sustained neurite outgrowth, cell-autonomous neuronal excitability (*i.e.*, the ability of cells to generate and propagate trains of electrical impulses) and functionality of the resulting electrical network. Moreover, these neurons were electrically active and had connected together to form an active neural network with functional synapses.

Ariano's group from Turin University showed [26] that multi-electrode arrays could be fabricated from diamond, and used these to measure the cell excitability of cultured rat hippocampal neurons and chick ciliary ganglia that had been grown on hydrogen- and oxygen-terminated diamond surfaces. They found that the cells survived, adhered and maintained their electrical properties (synaptic activity, ion channel availability, and Ca^{2+} signals during neuronal stimulation) for days, provided that mixtures of adhesion molecules (such as poly-D-lysine, poly-DL-ornithine, or laminin) were used to anchor the cells on the diamond surface. The importance of these recent publications is highlighted by the 2 recent European 'DREAMS' projects [6] which funded a number of European groups to work on cell/diamond interfaces.

Along with growth of neurons, neuronal patterning techniques provide the opportunity to control neuronal projections and direct specific neural pathways to particular electrodes. Directed neuronal growth, by infusing neurotrophic growth factors or by placing pieces of sciatic nerve into glass microelectrodes, has been shown to promote the ingrowth of neuronal processes *in vivo* [27]. In these studies, the recorded signal:noise ratios (SNR) were often 5–10 times higher than those obtained with wire and silicon electrode arrays [16,27]. A variety of techniques have been developed for patterning cellular growth. The most commonly used methods include micro-contact printing (MCP), photolithography, inkjet printing and stencil-assisted patterning techniques. These techniques generally involve patterning proteins or factors that either attract (extra-cellular matrix proteins, such as poly-lysine) or repel (anti-biofouling agents) cellular attachment, although some techniques have focused on the direct placement of cells [28,29]. MCP has been used to produce patterns of laminin and poly-lysine for spatially directing the growth of primary hippocampal and cortical neurons on many different substrates, including glass, polystyrene and diamond [24,30–32]. However, MCP has some limitations. For example, the recipient substrate has to be planar and should not be softer than the stamp material. The concentrations of transferred protein may also vary across the pattern, depending on the degree of contact between areas of the stamp and the recipient substrate, which could all be affected by a non-homogenous surface roughness, topology or wettability.

An alternative method, drop-on-demand inkjet printing, has been widely used for depositing patterns of liquids and suspensions onto surfaces at micrometre-scale resolutions (*e.g.* 2–50 μm spot sizes [33]). Although photolithography and MCP techniques can provide better spatial resolution, they require the construction of an unalterable master pattern and so lack the programmable versatility of inkjet printing. Additionally, the low cost and pattern reproducibility make it a very useful fabrication technique. For example, inkjet-printed poly-D-lysine and collagen patterns (with dot resolutions of 65 μm) have been used to direct the adhesion of primary rat hippocampal and cortical neurons, with no obvious detriment to the electrophysiology of the cells [34].

Another method that offers some of the same advantages as inkjet printing is laser micro-machining. In this technique, a high-power focused laser beam is used to directly write patterns into the substrate surface, or into the polymer adhesion layer upon which the cells subsequently grow. Modern laser patterning machines are computer controlled and allow patterns of almost any design or complexity to be etched into a surface with a resolution of a few microns.

This paper describes results from a small pilot project to demonstrate that rat neurons could be successfully cultured on hydrogenated and/or oxygenated diamond surfaces using a modified version of the UCL procedure. The aim was to identify characteristics of the diamond surface, such as surface termination type, boron doping level, crystal morphology and smoothness, that help or hinder neuron growth. A second aim was to highlight laser micro-machining as being a novel, high-resolution method for patterning the diamond surfaces, and thereby spatially direct neuronal growth along predesigned pathways.

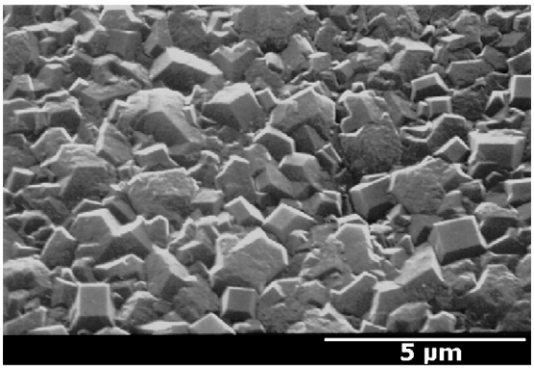

Fig. 1. Scanning electron micrograph of a CVD diamond film used in this study.

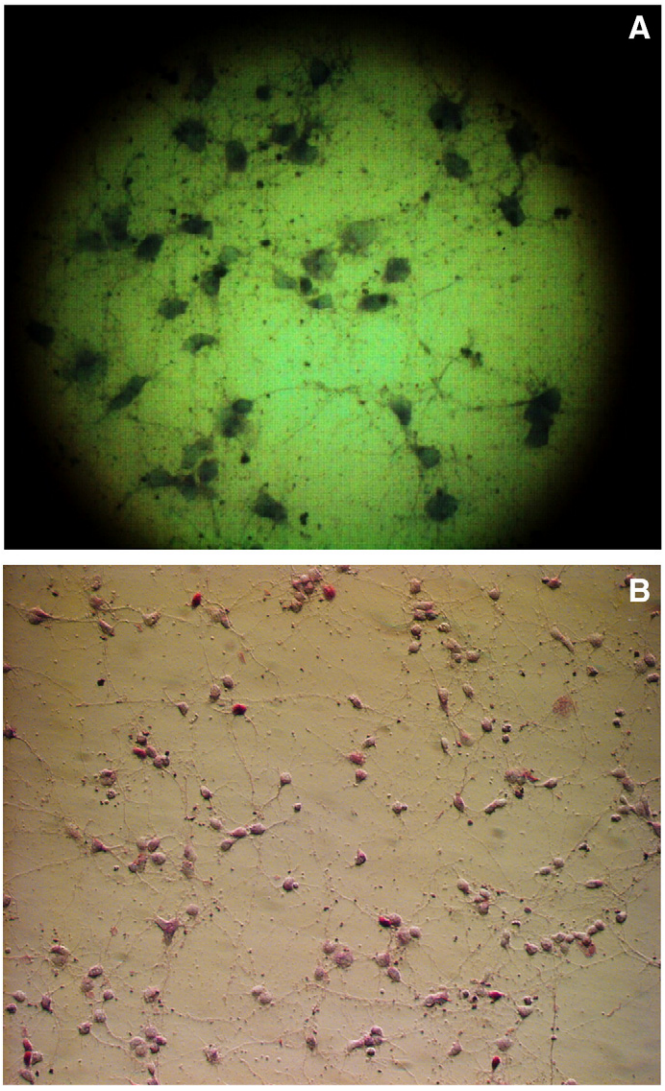

Fig. 2. Example images of stained neurons used to determine the cell counts.(a) Neurons on a CVD diamond surface. (b) Neurons on the TCPS control.

2. Experimental methods

(a) Diamond substrates: Boron-doped diamond films were grown on Si (100) substrates in a standard hot-filament chemical vapour deposition (CVD) reactor using $CH_4/H_2 + B_2H_6$ as process gases at a pressure of 20 Torr. The resulting diamond films were around 3–4 μm thick over an area ~1 cm^2. The films were microcrystalline, with a facetted morphology having crystal sizes on the surface ~1 μm (see Fig. 1). Varying the B content in the gas mixture, and hence in the films, allowed the conductivity of the films to be controllably altered between extremely insulating (resistivities of several MΩ cm) to near metallic (resistivities of a few Ω cm). After deposition, the film surfaces were either left as grown (*i.e.* a hydrogenated surface) or oxidised using UV-irradiation/ozone to increase the surface hydrophilicity. Tissue culture polystyrene (TCPS) plus commercial unpolished polycrystalline (crystal size ~20 μm) and polished single-crystal CVD diamond (bought from Element Six, Ltd) were used as control samples.
In order to create the thin adhesion layer required for the neurons to stick to the diamond surface, samples were initially coated with poly-D-lysine (0.1 mg ml^{-1}) for 1 hour, washed three times in deionised water and dried in air. Before addition of neurons, samples were sterilised by immersing the substrates in a 10% (v/v) penicillin/streptomycin/amphotericin B(PSA) solution, washed in deionised water three times and dried.

(b) Laser patterning: A Nd:YAG laser (532 nm) micromachining system (Alpha, Oxford Lasers, UK) focused to a spot diameter of ~10 μm was used to etch patterns into the surface of the poly-lysine-coated boron-doped CVD diamond substrates. A laser power of 0.8 W was found sufficient to completely ablate the diamond film exposing the Si surface beneath. A sequence of 10 connecting rectangles (100 μm width, 1000 μm length) was created which were visible under an optical microscope.

(c) Cortical neurons: Neuron-rich cultures were produced by isolating the cerebral cortices from the pups of embryonic day-18 (E18) Wistar rats. Standard tissue-culture protocols [35] were used to grow the cells, and then they were seeded at a concentration of 1×10^5 cells per well onto the boron-doped diamond substrates. A serum-free nutrient medium (Neurobasal) supplemented with 2% B-27, L-glutamine, glutamic acid and penicillin/streptomycin was used to feed the cells. The cell cultures were then placed in an incubator at 37 °C/5%CO_2 and fed at 3-day intervals by replacement of half of the medium (minus glutamic acid). Cells were then fixed at 3 days *in vitro* (DIV) with 4% *para*formaldehyde for 20 min, washed, stained with toluidine blue, and photographed using a Leica imaging system. Software then automatically counted the number of adhered cells per mm^2, and averages were made of 20 measurements.

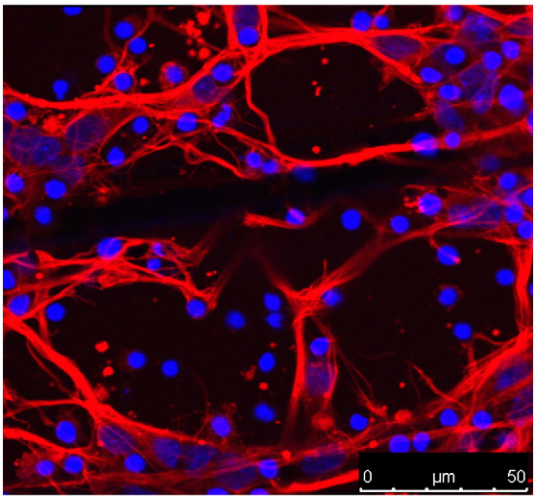

Fig. 4. An example of a stained sample showing healthy, spherical blue nuclei and red dendrites, but *no* green glia.

Immunocytochemistry was used to provide more information about the types of neurons growing and their structure. Primary monoclonal mouse antibodies to beta-III-tubulin (ABCAM, UK) were added to the fixed cells, which bound to specific proteins. Then secondary antibodies were added which contain fluorescent markers and they bound to the corresponding primary antibody. A blue fluorescent marker (*Hoescht*) which binds to DNA was used to reveal cell nuclei, while a red fluorescent marker (*Beta-III-Tubulin*) was used

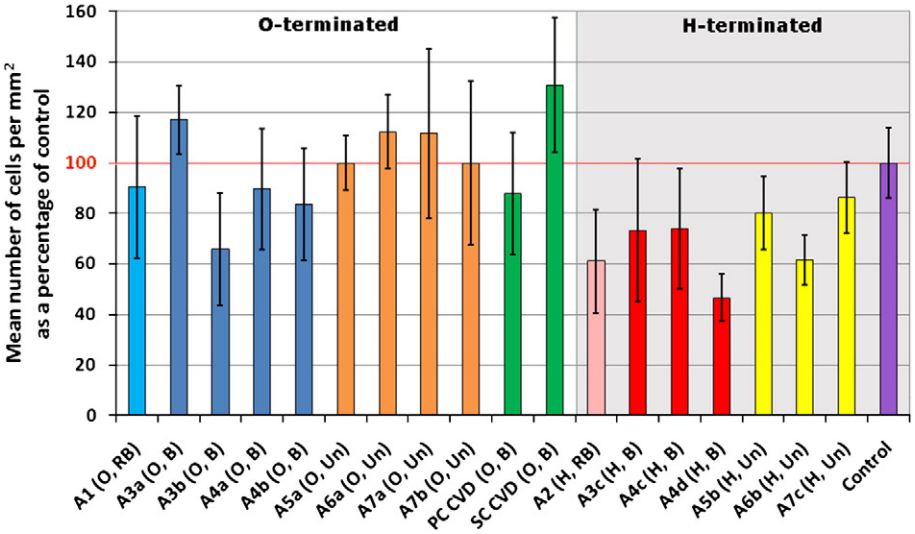

Fig. 3. Average number of neurons per mm^2 recorded as a percentage of counts on the control sample (TCPS), with their standard deviation values. CVD samples were coded from A1-A7 and then each was divided into 4 sections (a-d), with each section undergoing a different post-deposition treatment. For example, A4a(O, B) and A4d(H, B) were originally part of the same sample deposited with the same B content, except that 'a' was then O-terminated while 'd' was H-terminated. Key: O = oxygen terminated, H = hydrogen terminated, RB = very lightly doped with B from the residual boron in the chamber with film resistivity of a few kΩ cm, B = heavily B doped with film resistivity of a few Ω cm, Un = undoped with film resistivity of a few MΩ cm, A1-A7 = microcrystalline CVD diamond films grown on Si, PC CVD = mechanical-grade CVD commercial film from Element Six, SC CVD = single-crystal CVD commercial film from Element Six, Control = TCPS control sample. Colour-coding of the bars has been used to aid the reader by grouping samples with similar properties, *e.g.* blue is for oxygen-terminated B-doped samples, orange for oxygen-terminated undoped samples, green for commercial bought samples, etc.

to reveal cell membranes and dendritic growth. A green marker (*GFAP*) was used to reveal glia. More details can be found in Ref. [22].

3. Results

(a) Cell counts: Fig. 2 shows examples of the images of the stained neurons used to determine the cell count values, whilst Fig. 3 shows the tabulated cell counts for different diamond surface terminations, B-doping level and type of diamond.
  (i) Effect of O/H termination: As reported by other workers [7,11], the O-terminated diamond surfaces showed a cell growth rate that was as good as or even better than the control sample, so long as there was a suitable adhesion layer (such as poly-lysine) present. With no poly-lysine layer present, cell growth was zero — with most neurons dying within a few days. This confirms that O-terminated CVD diamond surfaces combined with an adhesion layer are an excellent medium upon which to culture neurons. Conversely, the H-terminated surfaces are only about 50-60% as good as the control.
  (ii) Effect of B-doping: There is no statistical difference between any of the samples with varying B-doping concentration. This shows that the B-doping level of the diamond is not a factor in the growth of neurons.
  (iii) Type of diamond: There was no significant difference between the two types of microcrystalline diamond, home-grown thin films (A1-A7) with smaller crystallite size, and thicker commercial diamond (PC CVD) with larger crystallite size. However, the single-crystal sample (SC CVD) shows the highest cell adhesion rate. The reason for this may be that this sample had been polished, and the smoother surface allows more uniform covering of oxygen groups and/or a better platform for the poly-lysine coating.

(b) Immunocytochemistry: Fluorescent markers were added to the neurons after 7 days *in vitro* (DIV). In Fig. 4, these markers show: blue (nuclei), red (cell membranes & dendritic growth), and green (glia cells). It is clear that after 7 DIV the neuronal growth is healthy. The cellular bodies are spherical (blue) and there is a large amount of strong prominent dendrite growth (red). No glia cells (green) are present, probably as a result of using a serum-free nutrient medium. These results confirm that O-terminated CVD diamond is able to support a functioning, healthy neural network.

(c) Laser patterning: Fig. 5 shows an electron micrograph of the laser-patterned surface before application of neurons. The diamond coating has been completely removed in the etched areas, and the underlying Si has locally melted and resolidified due to the action of the laser. In contrast, in unetched areas the poly-lysine-coated diamond layer remains pristine. After the neurons were applied, Fig. 6 shows that the neurons adhered almost exclusively to the areas which were not laser etched, *i.e.* which still had the poly-lysine-coated diamond layer present. Moreover, dendritic growth was also mostly confined to these non-etched areas, although there were some areas of cross-over where dendritic growth bridged the gaps between features.

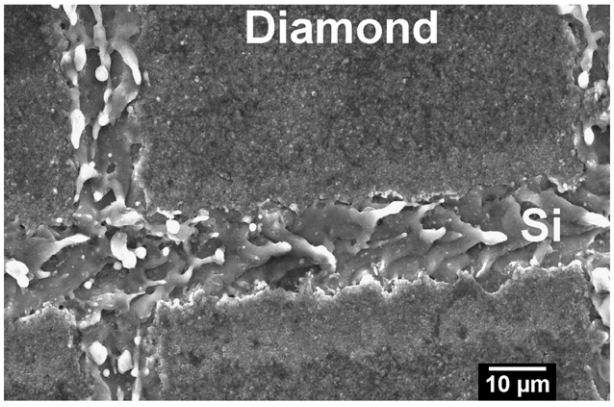

Fig. 5. Scanning electron micrograph of the substrate surface after laser patterning showing areas of pristine unetched diamond and stripes of the exposed Si that has melted & resolidified.

4. Conclusions

Oxygen-terminated CVD diamond is an excellent substrate upon which to grow neurons, and can support a functioning, healthy neural network. The survival rate and lifetime of neurons on O-terminated diamond can equal or exceed that of standard biological substrates such as TCPS. However, a cationic polymer anchor layer, such as poly-D-lysine, is needed to keep the neurons attached to the surface, while a nutrient medium is required to keep them alive. H-terminated diamond also allows growth of neurons in the same manner, however the lifetime and survival rates were typically ~70% of those on the

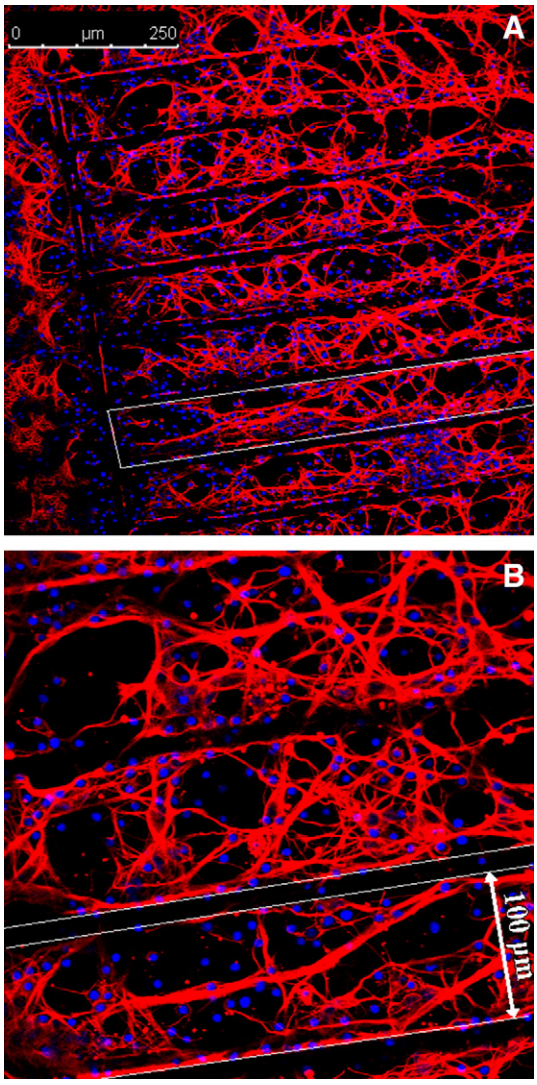

Fig. 6. Fluorescence microscope images of neuron growth on laser-patterned O-terminated CVD diamond after staining, shown at (a) low and (b) high magnification. The laser-patterned rectangles have been highlighted by white lines. Nuclei are stained blue, dendritic growth is stained red.

TCPS control substrate. The difference between the neuron behaviour on the two types of diamond termination is probably related to the hydrophobicity and hydrophilicity of the H- and O-terminated surfaces, respectively.

The B-doping level of the diamond does not affect neuron growth. This is an important finding. Boron is a potentially poisonous element for many cells, so it is crucial to discover that the boron embedded within the diamond lattice does not damage or kill any cells on the surface. This is a highly encouraging result if B-doped diamond is to be used in BCIs.

However, the surface roughness may be an issue, with smooth single-crystal diamond producing typically a ~20–30% increase in neuron survival rate over polycrystalline substrates. This may be because the smoother surface allows more uniform covering of oxygen groups and/or a better platform for the poly-D-lysine coating, although more work needs to be done to confirm this.

Laser patterning to remove the diamond layer is a simple way to promote selective area growth of neurons and thereby form designed neural pathways. Use of a serum-free nutrient medium, such as Neurobasal, ensures that the neurons do not spread beyond the areas delimited by the patterned poly-lysine. Because laser ablation techniques have been reported to reach sub-micron resolutions, we would predict that this technique has the potential to be used to pattern individual neuritic processes, as well as single-cell somata. Reducing the laser intensity should, in future, allow selective ablation of just the poly-lysine adhesion layer, leaving the diamond intact. This may provide another route to patterned neuron growth.

It is clear that this area of research is still very new, and much more work needs to be done. In particular the effect of the diamond surface roughness upon neuron growth needs more systematic investigation. Electrical measurements of action potentials from neurons on diamond are also being undertaken to determine whether the synapses are functional and remain healthy, and to ensure the viability of the active neural network. Although the results are still preliminary, we believe that these microtailored substrates could benefit the long-term performance of BCIs by providing improved levels of neuronal connectivity, better organisation of the neuron-electrode interface and higher resistance to degradation. Moreover, this work will inform and have further applications in neural-network research, investigating inflammatory-cell responses and in the development of nerve and brain repair scaffolds.

Acknowledgements

The authors wish to thank Jo Howarth and Helen Scott for help with the clinical dissections, and James Smith and Raquel Vaz for assistance with the laser patterning.

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