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# Long-term culture of pluripotent stem-cell-derived human neurons on diamond – A substrate for neurodegeneration research and therapy

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ABSTRACT

Brain Computer Interfaces (BCI) currently represent a field of intense research aimed both at understanding neural circuit physiology and at providing functional therapy for traumatic or degenerative neurological conditions. Due to its chemical inertness, biocompatibility and stability, diamond is currently being actively investigated as a potential substrate material for culturing cells and for use as the electrically active component of a neural sensor. Here we provide a protocol for the differentiation of mature, electrically active neurons on microcrystalline synthetic thin-film diamond substrates starting from undifferentiated pluripotent stem cells. Furthermore, we investigate the optimal characteristics of the diamond microstructure for long-term neuronal sustainability. We also analyze the effect of boron as a dopant for such a culture. We found that the diamond crystalline structure has a significant influence on the neuronal culture unlike the boron doping. Specifically, small diamond microcrystals promote higher neurite density formation. We find that boron incorporated into the diamond does not influence the neurite density and has no deleterious effect on cell survival.



## 1. Introduction

In the past 10 years diamond has emerged as a biomaterial targeted towards neural implants [1–3], driven by the maturing technology of chemical vapour deposition (CVD) which allows thin films of diamond to be deposited relatively inexpensively over areas of many square cm [4,5]. Inherent to diamond are a number of characteristics which make it a desirable substrate for tissue implantation. Diamond is not chemically reactive, it is not immunogenic and it is stable over extremely long periods of time. This means that a diamond prosthesis will very likely survive the patient [6–10]. Considering diamond's extreme rigidity, such prostheses would be unsuitable for implantation into moving parts of the body and, would be best suited for Brain Computer Interfaces (BCI). An attractive feature of diamond is that while it is electrically insulating in its pure form, it can be made conductive by doping with atoms that could act either as donors or acceptors of electrons and thus, able to conduct electrical impulses from/to adjacent cells [11,12]. The advantage of this method is that the conductive and insulating part of an electrode would be made of the same bioinert material. Rodent and human neurons, derived from neural progenitors and neural stem cells, have been successfully cultured on diamond or diamond like carbon substrates [1,2,13–17], and have been reported to grow along pre-patterned diamond or diamond like carbon stripes to form 2D neural networks [18]. Recently, a boron-doped diamond probe implanted in rodents in contact with the cerebral cortex for six months has been shown to be free of any major histological or physiological side-effects [3], and a diamond retinal prosthesis has been proposed [19]. Furthermore, nitrogen included ultrananocrystalline diamond and boron doped diamond

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have been shown to induce less fibrotic encapsulation than silicon [20].

Of note, diamond's unusual properties make it a potentially much more useful substrate than a simple replacement for the current titanium cortical implants. Specifically two "*in vitro*" lines of investigation are currently open. One involves the study of the direct interaction of neurons with the substrate, both mechanically and electrically, towards optimization of the probes of the future. The other involves the study of neuronal aging and degeneration in an electro-physiological context on a diamond multi-electrode array (MEA). Both these lines of investigation are dependent on the long-term culture of specific types of mature human neurons. However, the extreme sensitivity to "*in vitro*" manipulation and very limited availability of human mature neurons, as well as the limited differentiation potential of neural progenitors, are significant constraining factors. Therefore, the ideal solution would be the "*in vitro*" generation of mature neurons from pluripotent stem cells.

Currently there are two sources of human pluripotent cells: epiblast-derived human embryonic stem cells (hESC) and adult cells converted "back to pluripotency", also termed human induced-pluripotent stem cells (hIPS). hIPS are pluripotent cells derived from adult donors which allow both "*in vitro*", highly reproducible studies of specific diseases, as well as transplantation back into the patient, free of host-versus-graft reaction.

Since neuronal differentiation from pluripotent cells reflects the long human development, any differentiation protocol would have to face the challenges of extended time in culture; *i.e.* over 100 days. Currently, human pluripotent cells are being differentiated into various neuronal subtypes in monolayer, on plastic and in defined chemical conditions. Following the successful culture of mammalian neural cells on diamond substrates [1,2,13–15,17,18] it could be inferred that differentiation towards neuronal fates from human pluripotent cells should also be possible on these substrates. However, the optimal conditions that would support human neurons in long-term culture are currently unknown.

Furthermore, cell response to diamond doped with boron, an electron acceptor, is also poorly understood. Boron is an element with dose-dependent toxicity. In laboratory animals, boron compounds have been found to be toxic only at very high doses [21,22], while in humans, studies of workers chronically exposed to relatively high levels, failed to demonstrate pathological consequences [23,24]. However, exposure to boron leads to accumulation in the brain [25] and, *in vitro* testing indicated that boron nanotubes are more toxic than carbon nanotubes to cells in culture [26].

We present here a protocol for differentiation of human IPS cells into cortical neurons on microcrystalline diamond and investigate the role of diamond structure in this process. We also show that diamond doped with boron, an electron acceptor, does not adversely influence either the differentiation process or the health and viability of the neurons.

## 2. Material and methods

### 2.1. Diamond characterization

Commercial diamond was purchased from Element Six, Ltd., either as a single-crystal plate (P2, 145-500-0055), or as a polycrystalline smooth-polished diamond plate (TM-CVD 145-500-0090). These were compared with the in-house produced diamond as previously described [4]. Briefly, a thin diamond film was deposited on a silicon wafer (Silicon Materials <100>) in a hot filament reactor using standard chemical vapour deposition (CVD) conditions (1%$CH_4$ in $H_2$, 2.67 kPa pressure, filament temperature 2400 K, substrate temperature ~1100 K, for 2–12 h). This produced an insulating, continuous microcrystalline diamond film at a rate of about 0.5 μm $h^{-1}$. For boron-doped diamond 2000 ppm diborane gas was added to the mixture which increased the conductivity of the diamond film to near-metallic [27].

All samples were then cleaned in concentrated nitric acid (68% v/v). The as-grown hydrogen-terminated diamond surface was then converted to a predominantly oxygen-terminated surface, as previously described [2], by exposing the sample to ozone for 20 min (UVO cleaner, Jetlight Co. 42A-220). Previous reports [2] have shown that oxygen-terminated diamond surfaces are preferable for cell survival.

To test the best diamond structure for neural culture we made use of a feature of diamond CVD that the size of the crystals produced depends on the deposition time. To that effect, we employed a hot filament reactor to grow polycrystalline diamond films for times ranging between 2 and 12 h (Fig. 1, and Suppl. Fig. 1). The supportive properties of the in-house diamond were compared with those of a polycrystalline smooth-polished diamond (Suppl. Fig. 2).

The diamond crystals were imaged by scanning electron microscopy (SEM), using a Quanta 400FEI system. To quantitatively assess the size of the crystals produced, two complementary methods were used: measurement of the average distance between the sharp peaks of 2 adjacent crystals, and counting the number of sharp peaks within an area of 1 $\mu m^2$ (Fig. 2). We speculated that these are the parameters with most biological significance, as the sharp corners of the crystal facets are the most conspicuous structural features a cell will encounter. The boron-doped diamond crystals (Suppl. Fig. 1) were slightly larger than the corresponding undoped crystals grown for the same amount of time. However, quantification indicated that although larger, the boron-doped diamond crystals were not large enough to match the size of the next tier of undoped diamond crystals (Suppl. Fig. 3).

### 2.2. Raman spectroscopy

The Raman spectra were measured at room temperature by means of a Renishaw spectrometer. The samples were excited using an argon-ion laser at $\lambda = 515.5$ nm. The laser power was set to 6 mW and the spot size to 15 μm. The spectra for undoped and B-doped diamond (Fig. 3) are consistent with high quality microcrystalline diamond films with little or no graphitic impurities [28].

### 2.3. ES/IPS cell culture

We set out to adapt the current protocols for differentiating human pluripotent cells towards neuronal lineages [29–31] to the specifics of a diamond substrate. Furthermore, a requirement for cells used for regenerative medicine is that they are cultured in fully defined conditions free of animal components (xeno-free). Human pluripotent cells are routinely cultured on a feeder layer of mouse embryonic fibroblasts (MEFs) in Knock-Out Serum Replacement (KOSR), which contains bovine serum albumin (BSA). Consequently, the culture conditions are neither defined nor xeno-free. A feeder-free system has been applied successfully (TeSR) [32]. However, this system uses Matrigel as a substrate (a poorly defined matrix of animal origin) and requires BSA. Recently, two fully defined, xeno-free systems became commercially available: Essential 8™ (Life-Technologies) [33] and, StemFit™ (Ajinomoto Co., Inc.) [34]. However, most protocols for neuronal differentiation from hIPS use KOSR-based media as the starting point.

Three pluripotent lines have been used for this study: ES-Shef6 (MRC Stem Cell Bank), IPS-MSU (Michigan State University) and IPS-NAS2 (Dr. Tilo Kunath laboratory) [35]. Cells were maintained in xeno-free and feeder-free E8 medium as recommended by the manufacturer (LifeTechnologies, A1517001). Briefly, cells were