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Development of *in vitro* 3D brain models on laser fabricated scaffolds

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Abstract: In this work, we present the design and implementation of a 3D scaffold platform that supports and promotes intricate neuronal network development. 3D scaffolds were produced by two-photon polymerization (2PP), a high-resolution 3D laser microstructuring technology, using the biocompatible and non-degradable photopolymer. Human iPSC-derived neuronal networks have developed and interconnected in a 3D environment shaped by vertically stacked scaffold layers. The iPSC-derived neuronal progenitor cells could develop into cortical projection neurons (CNPs) of all six layers, different types of inhibitory neurons and glia. The network showed strong neuronal spontaneous activity that combined individual and collective signaling events. Our results highlight advanced microstructured 3D scaffolds as a reliable platform for 3D in vitro modelling of neuronal functions.

I. Introduction

The goal of most current tissue models, including neuronal tissue, is to establish systems which can be used to study pharmacology, network formation, pathology, or any combination thereof [1]. Truly 3D polymeric scaffold platforms with complex and predictable architecture represent a powerful tool in developing instructive milieu for *in vitro* cell accommodation and development of tissue with certain functional properties. The highest level of flexibility and reproducibility in terms of structural design can be achieved using the two-photon polymerization technique (2PP). The aim of the present study is to establish a reproducible human iPSC-derived neuronal 3D *in vitro* model using advanced 2PP laser technique for the fabrication of the 3D scaffold platform (Fig. 1a). We investigated the ability of human iPSC-derived neural progenitor cells (NSCs), grown and differentiated on these 3D scaffolds, to develop mature and functional neuronal networks. With this culture system, we demonstrated that 3D microstructured scaffolds can dictate several important characteristics of NSCs derived neuronal networks [2].

II. Material and methods

3D scaffolds were fabricated from the commercially available biocompatible resin Dental-LT-Clear (Formlabs, Berlin, Germany). 9 mm round scaffolds were produced by the two-photon polymerization (2PP) with a BioScaffolder+ system (Laser nanoFab GmbH). 3D scaffold architecture was composed out of three 80 µm high cylinder layers (Fig. 1 a), with inner pore diameter of 240 µm and 30 µm cylinder wall thickness. Human male iPSC-derived neuronal stem cells (NSCs) were provided by Axol Biosciences Ltd. (Cambridge, UK) and cultured according to supplier instructions. 3D cultures were monitored over 120 days and compared to 2D cultures on the same material and glass coverslips.

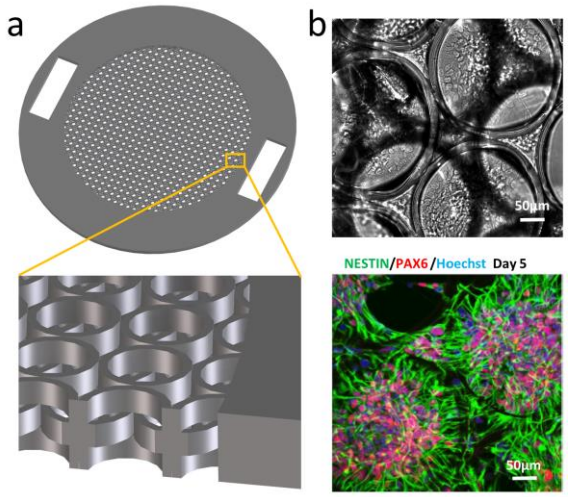

Figure 1: a) Top: CAD design of a 3D scaffold, showing the honeycomb-like pore pattern; b) Top: phase contrast imaging showing uniform cell distributed within the scaffold 5 days after seeding. Bottom: NSCs are well accommodated in the pores of the scaffold expressing cytoskeletal and nuclear markers characteristic of cortical stem cells (Nestin and PAX6).

III. Results and discussion

Our laser produced 3D scaffolds enabled long term culture of human iPSC-derived neuronal networks over 120 days. In contrast, 2D control cultures —prepared as a flat layer of the same material and cultured identically— could be

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maintained only up to 50 days. After that, irreversible cell delamination began to occur. *In vitro* functional maturation of the neuronal network (synaptogenesis) was confirmed starting from the day 30 of culture (Fig. 2 a). Using immunofluorescence analysis, we found a mixed population of glutamatergic excitatory and GABAergic inhibitory neurons, which are crucial to both, the development and function of the cerebral cortex. 3D long-term culture maintenance, therefore, facilitated iPSCs-derived neuronal progenitors to develop into functional neuron-glia networks consisting of cortical projection neurons of all six cortical layers, different types of interneurons and astrocytes (FIG. 2).

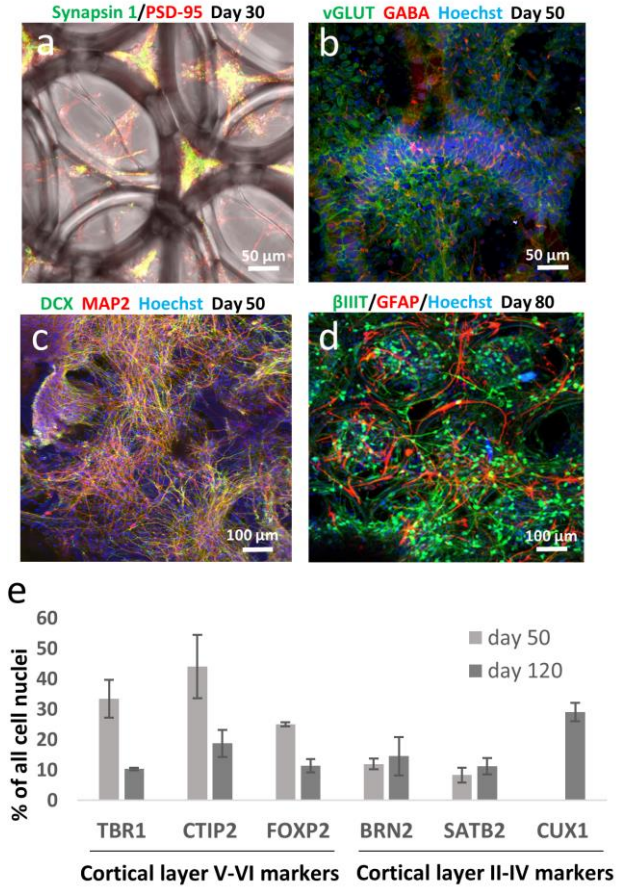

Figure 2: Immunofluorescence analysis of neuronal and astrocyte markers over the course of neuronal differentiation: a) at day 30 formation of functional synapses was identified by the analysis of pre- and postsynaptic protein complexes through Synapsin 1 and PSD-95, respectively; b) development of specific excitatory vGLUT1+ and inhibitory GABA+ neuronal cells was observed at day 50; c) developing network stained with early marker of neuronal identity DCX and mature neuronal marker MAP2; d) rich and well interconnected neuronal network with βIIIT+ neurons was also characterized by the presence of GFAP+ astrocytes; e) proportions of different classes of cortical projection neurons at days 50 and 120 relative to total cell population.

Calcium imaging allowed for the quantification of functional network connectivity, indicating that scaffold structure promotes the development of local connectivity of neuronal circuits (FIG. 3). The demonstrated capacity to modulate neuronal network connectivity in tailored structures already paves the way towards future explorations with more complex and functional scaffold designs. The ability of NSCs to generate different types of neuronal cells, when grown on the 3D scaffold platform, enables future functional studies of the human cortex development. Thus, our 3D culture represents a novel platform with a high potential for the development of physiological *in vitro* models of human neuronal networks.

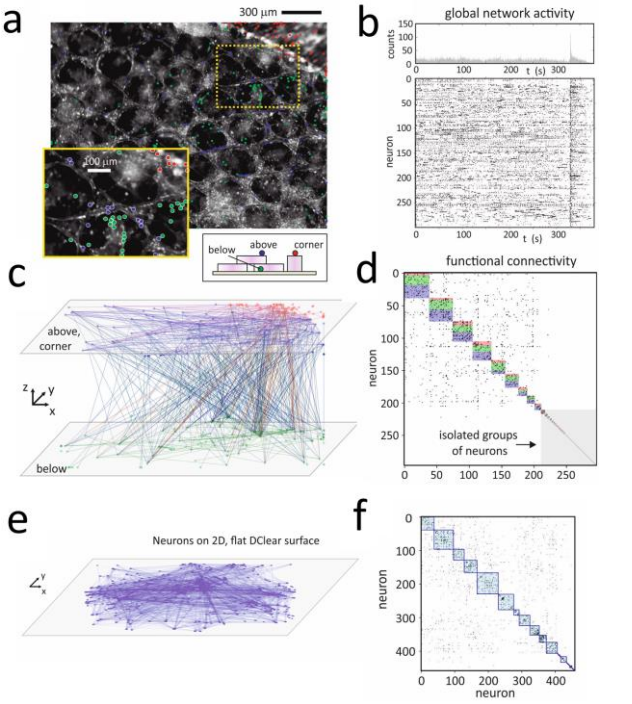

Figure 3: Analysis of calcium imaging data of neuronal networks grown and differentiated on the 3D scaffolds for 50 days. a) Regions of interest positions (neurons) as colored dots; Enlarged insert and schematic representation of neurons assigned to different layer categories; b) raster plot of spike times (bottom) and global network activity (top); c) graphical representation of the 3D network functional connectivity and (d) effective connectivity matrix ordered by functional community; (e-f) comparative Effective connectivity of a control culture grown in a 2D conditions.

IV. Conclusions

The proposed 3D scaffold-based culture system will allow new insights into *in vitro* modelling neurological diseases with NSCs and will also enable the creation of engineered tissue constructs for studying innervation of different types of tissues, such as skin, muscle, bone and cornea.

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AUTHOR'S STATEMENT

Conflict of interest: Authors state no conflict of interest. Informed consent: Informed consent has been obtained from all individuals included in this study.

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