

Engineering Human Neural Networks: Controlling Cell Patterning and Connectivity

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Abstract

Restorative treatments for diseases affecting the central nervous system (CNS) are difficult to develop, due to the complexity of CNS tissues and transferability issues with results from costly animal models. There is an urgent need to produce reliable, complex culture models to develop effective treatments. Such models require controlled interfaces and relevant dense neural cultures. In this thesis, techniques are developed to prepare and investigate scaffolds enabling complex structured neural model fabrication.

To facilitate development of an interface layer, neural culture on polycarbonate track-etched membranes demonstrated that the growth of neural processes through pores required confluent cultures. To direct low density cultures, funnel-shaped pores were machined into glass coverslips, and neurite interaction with angled pore edges was analysed. Live-imaging results showed that neurites more often crossed shallower edges, and retreated from steeper edges.

Concerning development of dense cultures, neural culture in non-granular hyaluronic acid (HA) hydrogels showed cell clustering and reduced neurite extension. A protocol adding secondary structure to the scaffold by granulating HA hydrogel was optimized, and cell viability and connectivity within the hydrogel were analysed. Cell viability in the granular hydrogel was comparable to the control, and there was improvement of network connectivity in granular hydrogels over non-granular counterparts.

Potential application to improve nerve graft technology motivated the design of an extrusion device that generates tertiary structure by interspersing cell-seeded and unseeded granular HA hydrogel, facilitating control of cell distribution and alignment within the scaffold. Tertiary extruded and non-extruded hydrogels were analysed, and distribution was maintained within the tertiary extruded hydrogel scaffold, without detriment to the cell functionality. It is hypothesized that additional guidance cues could be added to the scaffolds to control cellular alignment.

Findings demonstrate the fabrication of structured scaffolds optimized for neural network growth, and highlight strategies that can be used in the production of *in vitro* neural models for complex CNS study.

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Acronyms and Symbols

List of Acronyms

2D	two-dimensional
3°	tertiary
3°EG	tertiary extruded hydrogel
3D	three-dimensional
8-br-cAMP	8-bromoadenosine 3', 5'-cyclic monophosphate
ANOVA	analysis of variance
ATCC	American Type Culture Collection
BDNF	brain-derived neurotrophic factor
CI	calcium imaging
CNS	central nervous system
CT	computed tomography
d	day
DIC	differential interference contrast
DMEM	Dulbecco's Modified Eagle's medium
DPSC	dental pulp stem cell
DRG	dorsal root ganglion
DWS	diffusing wave spectroscopy
ECACC	European Collection of Authenticated Cell Cultures
ECM	extracellular matrix
FBS	fetal bovine serum
GFP	green fluorescent protein
GG	granular hydrogel
HA	hyaluronic acid
HF	hydrofluoric acid

M	minutes
MEA	microelectrode array
MRI	magnetic resonance imaging
MSC	mesenchymal stem cell
N	number of observations/measurements in graph
N3°EG	non-tertiary extruded hydrogel
NBM	neuronal base medium
NEAA	non-essential amino acids
NGC	nerve graft conduit
NGF	nerve growth factor
NGG	non-granular hydrogel
NIH	National Institutes of Health
NS	non-significance
OGB-1	Oregon Green 488 BAPTA-1
P/S	penicillin with streptomycin
PBS	phosphate buffered saline
PC	patch clamp
PCL	polycaprolactone
PDMS	polydimethylsiloxane
PEG	polyethylene glycol
PET	polyethylene terephthalate
PGA	polyglycolic acid
PGC	post-granulation crosslinker
PGS	polyglycerol sebacate
PLA	polylactic acid
PLGA	poly lactide co-glycolide
PLLA	poly (L-lactide)

List of Acronyms

PNS	peripheral nervous system
PS	polystyrene
RA	all-trans retinoic acid
Ra	mean roughness
RGD	arginylglycylaspartic acid
RPMI	Roswell Park Memorial Institute
Rq	root mean square roughness
Rz	mean roughness depth
TCP	tissue culture plastic
TEM	transmission electron microscopy
TRITC	tetramethylrhodamine
v/v	volume concentration
VEGF	vascular endothelial growth factor
w/v	mass concentration

List of Symbols

Units

°	degree
°C	degree Celsius
kg	kilogram
l	litre
M	molar concentration
m	metre
min	minute
Pa	pascal
s	second

Unit Prefixes

c	centi, 10^{-2}
m	milli, 10^{-3}
μ	micro, 10^{-6}
n	nano, 10^{-9}

Physical Symbols

G'	storage modulus
G''	loss modulus
t	time

Mathematical Constants

Π	pi
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Chapter 1: Introduction

1.1. Motivation

With an aging population, stroke and other diseases affecting the CNS are becoming the leading cause of long term disability. The cost to individuals, carers, healthcare systems, and the society is only set to increase. However, due to the low regenerative potential of the CNS, few restorative treatments exist. Furthermore, the complexity of the CNS tissues necessitate the use of animal models that are costly and require complex study designs, which may produce results with issues of repeatability and transferability to human CNS tissue. There is an urgent need to produce reliable complex culture models to aid in the development of effective treatments. Such models can be targeted using controlled interfaces which communicate with relevant dense neural cultures. This thesis is therefore motivated to investigate development of scaffold structures that can create the desired human neural networks for *in vitro* study.

Tissues in the CNS are highly organized, and representative tissue engineered *in vitro* models require multiple orders of structural organization of the scaffolds to direct cell positioning and migration for tissue formation. Three techniques to form scaffold structures are explored to determine the feasibility in preparing organized neural cultures: microfabrication to form funnel-shaped micropore structure, granulation to form granular hydrogel structure, and extrusion to form tertiary extruded granular hydrogel structure.

Motivating microfabrication of the funnel-shaped micropore structure is the potential for a neural network to be used as an interface system to an *in vitro* representative neural tissue construct, developed as a biological compartmental model. The generated neural interface is intended to facilitate improved targeted interactions via neurites extended from the interface layer into the neural tissue model. Electrode arrays have been previously used in this way to input spatially targeted stimulation into tissue models, however these arrays often become less effective over time due to the foreign body response [1]–[3]. Microporous

membranes have been used for cell culture and neural culture, and there is potential for using the microporous membrane to develop a low-density organized neural network which can interface with the representative *in vitro* neural model, reducing the chance of decreased function of the stimulation connections over time. A separate cell layer connected with the representative model, functions as a targeted signal initiator in the tissue model. Such a neural interface prepared through cell culture on arrayed, engineered pore shapes in the microporous scaffold, directs the growth and separation of neurites from their cell bodies on the top-side of the scaffold, to connect and communicate with the cell bodies of the representative model on the underside of the scaffold.

To facilitate the development of these neural interfaces, neurite interaction with hard materials and topographical surfaces has been investigated. Shallow angular topography is used to direct the growth of neuron processes into and through the micropores, and a microfabrication technique is proposed to create the angled topography. The investigation of the limit of angular restriction to the neuron process is used to discover neurite and growth cone interaction with topographical surfaces. Microfabrication as a structural technique has the potential to slow the loss of patterning and maintain precise positioning of the neurons and neurites in the interface layer, and therefore maintain the specificity of targeting of the layer. Currently, the methods of cellular patterning are limited to soft lithography, bio-printing and contact stamping of proteins. While this creates a short-term pattern for cell growth on a two-dimensional surface, with time the cells may extend beyond the intended pattern due to the production of the cells' own extracellular matrix. Should the neurons interact with the microporous surfaces in the way expected, given the hypothesis that the growth cones of neurites are selective in motion across angled surfaces, it will provide an alternative cell patterning method with the ability to create longer term network constructs.

Motivating the granulation of hydrogel structure is the postulation that *in vitro* cell culture in three-dimensional hydrogels may create more representative neural tissue models. *In vitro* cell culture has long been dominated with the use of two-dimensional, tissue culture plastic as the scaffold, and recently, three-dimensional scaffolds have been investigated for preparation of more representative *in vitro* bulk parenchymal tissue [4], [5]. Hyaluronic acid hydrogels have been used for cell culture, but the crosslinked material is too stiff for the formation of connected neural networks. To overcome this issue, a granulated hydrogel, engineered to contain areas of lower stiffness for proliferation of neurons and extension of neurites, is investigated to determine degree of formation of connected networks without sacrificing the long-term stability of the hydrogel scaffold.

To facilitate development of dense neural structures required for representative neural tissue models, neural network formation within soft materials in the form of three-dimensional hydrogel scaffolds is investigated. Focus is on the optimization of a hydrogel manipulated by granulation to add a secondary structural level, to improve the scaffold for use with neural networks. Granulation as a structural technique has the potential to improve cell viability and network formation within the hydrogel, and modification of the crosslinking protocol to add a post-granulation crosslinking step is hypothesized to maintain the scaffold stability. Neural clusters are often observed in non-granular hydrogel scaffolds, which creates a non-representative three-dimensional tissue model. Should granulation of the hydrogel allow for more connected neural networks, this development can provide improved three-dimensional scaffolds for brain tissue engineering of *in vitro* “brain on a bench” constructs.

Motivating the extrusion of granular hydrogel structure is the development of a granular hydrogel with capabilities for culturing improved *in vitro* neural cultures which gives rise to the potential for application towards modifying artificial nerve graft technology. For this approach, the granular hydrogel is further modified, adding another level of structural

organization to produce a tubular granular scaffold, with the intention of developing constructs for nerve graft replacement systems. A hydrogel scaffold which maintains spatially distributed cell network with unidirectionally aligned neurites would function as a filler material for current medically approved nerve grafts which consist of empty tubing [6]–[8].

To facilitate development of a hydrogel filler for improved nerve graft conduits, a tertiary structural level is added to the granular hydrogel to control neural distribution and neurite alignment within the structured hydrogel. Extrusion as a structural technique has the potential to direct cell positioning and neurite alignment within the hydrogel, facilitating the application towards nerve graft filler material. Current medically approved artificial nerve grafts are limited to empty casings of biodegradable materials such as polyglycolic acid (PGA), polylactic acid (PLA), and poly lactide co-glycolide (PLGA), and these casings are successful in promoting nerve regeneration but only up to about 30 mm length nerve damages. Should the tertiary structured hydrogel direct the growth of axons in an aligned manner, it may provide an improvement to the grafting method by being able to extend the length of successful artificial grafting for nerve regeneration.

In summary, the neural network formed on the microporous scaffold, combined with the neural networks formed within either the granular hydrogel scaffold or the aligned hydrogel scaffold, form two versions of a multi-layer *in vitro* neural tissue model. This compartmental tissue model consists of a cellular neural interface (figure 1.1A) either for targeted stimulation of bulk parenchymal *in vitro* neural tissue with improved network connectivity (figure 1.1B) or for delivery of aligned and spatially distributed axons, separated from their neuron bodies by the microporous scaffold, into the tubular granular scaffold, replicating the *in vivo* regenerative environment of a damaged nerve (figure 1.1C). The *in vitro* tissue models and the structural techniques used to manufacture them have potential for a number

of medically related applications including neural disease model development, drug efficiency studies, and artificial nerve graft technology improvement.

1.2. Thesis Aim

This thesis has an overall aim of *developing techniques to form scaffolds enabling complex structured neural model fabrication*. The work is divided into three sub-aims: using microfabrication to guide neurite growth into funnel-shaped micropores, developing granulation techniques to improve the biocompatibility and three-dimensional (3D) network formation within a hyaluronic acid hydrogel, and designing a tertiary extrusion device to direct cell distribution and alignment by preparing a structured granular hyaluronic acid hydrogel scaffold. These sub-aims examine three levels of structural organization: primary, two-dimensional (2D) structure of the funnel-shaped micropores, secondary, three-dimensional structure of the granular hydrogel, and tertiary, ordered three-dimensional structure in the extruded granular hydrogel.

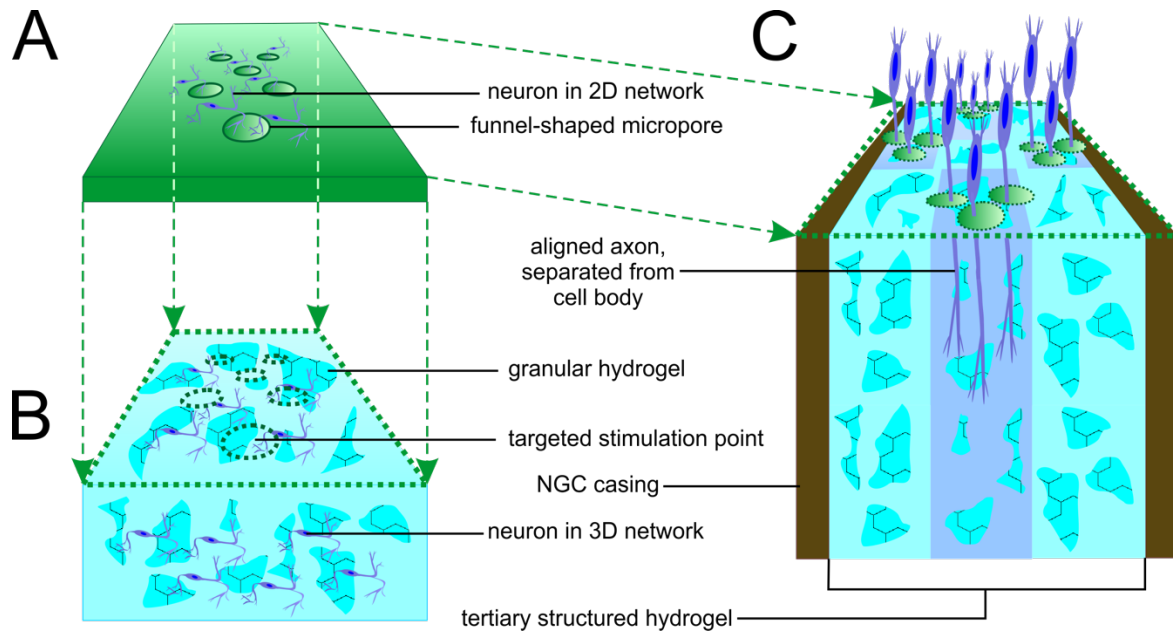


Figure 1.1. Schematics of the three investigated scaffolds. The flat, primary structured neural interface with a two-dimensional (2D) neural network (A), can either facilitate targeted stimulation of the secondary structured granular hydrogel scaffold forming a representative three-dimensional (3D) neural network (B), or direct growth of axons into the aligned hydrogel scaffold with added tertiary structure to model the improved artificial nerve graft conduit (NGC) (C).

1.3. Research Objectives

Research objectives are as follows:

- I. Using microfabrication to guide neurite growth into funnel-shaped micropores
 - a. Observe the base-line neural interaction with polycarbonate track-etched membranes at high magnification
 - b. Quantify the relationship between seeding density and neurite progression through polycarbonate track-etched membranes
 - c. Optimize the laser microfabrication, hydrofluoric acid etching, and surface smoothing protocols to prepare funnel-shaped micropores in glass coverslips
 - d. Characterize slope angle, funnel diameter, and surface roughness for prepared funnel-shaped micropores
 - e. Quantify the relationship between slope angle and neurite interaction with funnel-shaped micropores
 - f. Determine feasibility of the protocol

- II. Developing granulation techniques to improve the biocompatibility and 3D network formation within a hyaluronic acid hydrogel scaffold
 - a. Assess cell viability and network connectivity within unmodified, non-granular hydrogel scaffolds
 - b. Characterize the granule size and swelling of hydrogel granules prepared using the modified hydrogel reconstitution and granulation protocol
 - c. Characterize and compare the bulk rheological properties of the non-granular hydrogel and the granular hydrogel
 - d. Optimize the scaffold stability of the granular hydrogel scaffold using post-granulation crosslinker

- e. Compare the cell viability and network connectivity within the non-granular hydrogel scaffold and granular hydrogel scaffold prepared using the modified hydrogel preparation and seeding protocol
- III. Designing a tertiary extrusion device to direct cell distribution and alignment within a structured granular hyaluronic acid hydrogel scaffold
- a. Design schematics of the tertiary extrusion device to deliver interspersed cellular and acellular granular hydrogel
 - b. Optimize the device design by observing simple fluid modelling results
 - c. Verify modelling results by characterizing the hydrogel distribution within two versions of the device
 - d. Compare cell distribution and cell alignment of SH-SY5Y cells seeded within the tertiary extruded and non-tertiary extruded hydrogel scaffolds
 - e. Compare neural activity of F11 cells seeded within the tertiary extruded and the non-tertiary extruded hydrogel scaffolds

1.4. Thesis Structure

The thesis is structured in order to highlight the three sub-aims. It commences with a *literature review* chapter covering previous work in the field of scaffold manipulation in 2D and 3D for the purposes of directing cell growth into engineered tissues. Following this, a combined *methods* chapter describes the protocols, experimental setups, and analyses used. Three *results and discussion* chapters outline the work carried out to fulfil the research objectives as previously described. Finally, the *conclusions and future work* chapter brings together the three sub-aims, describing the contributions of this thesis to the field and proposing further investigations to continue improvements towards the intended applications.

Chapter 2: Literature Review

2.1. Tissue Engineering

Tissue engineering is a branch of biomedical engineering that focuses on recreating the *in vivo* environment for the culture of cellular material. The main goals of this field are to recreate human tissues and develop and improve technologies for clinical applications. Specific motivations range from the development of *in vitro* tissue models for disease or drug investigations, to the development of improved devices for *in vivo* tissue regeneration applications. There are two main focuses in developing an engineered tissue: the scaffold, and the cellular material. Cells are selected considering their ability to form networks and tissues with characteristics representative of the *in vivo* tissue. For the cellular material to differentiate and proliferate in specific ways, two-dimensional and three-dimensional scaffolds have been developed. These scaffolds have been optimized to have appropriate material stiffness for the cells to attach sufficiently, and porosity that allows room for the cells to grow and proliferate, while receiving adequate concentrations of chemicals required for this cellular growth. For different tissue types, there are a different range of factors to investigate, and there are very different directions that have been proposed to recreate the *in vivo* cellular environment. This project uses porous membranes and structured hydrogel scaffolds, with the goal of recreating an environment suitable for the culture of human brain and nerve tissue.

2.2. Investigating *in vitro* Neural Cultures

One goal of developing an engineered *in vitro* tissue model is to allow invasive study to be carried out on living human cell networks. This bridges a gap in research as it is not possible to perform such an invasive investigation in live human brain and nerve tissue. Current investigative procedures for use on the living human brain involve computed tomography (CT) scans (figure 2.1A) and magnetic resonance imaging (MRI) (figure 2.1B). These

procedures give information about the regions of the brain, and it is not possible to resolve detailed information about the neuron to neuron communication processes.

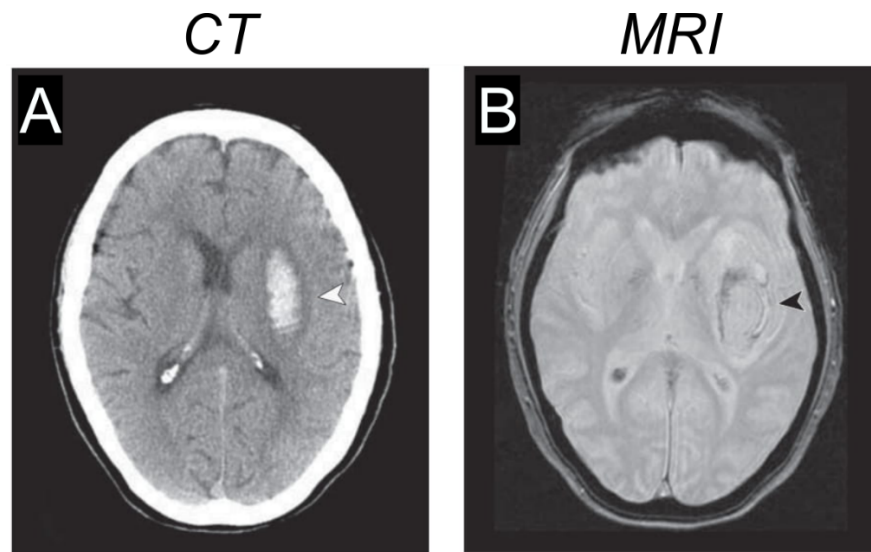


Figure 2.1. Images showing brain sections captured using computed tomography (CT) (A) and magnetic resonance imaging (MRI) (B). Arrowheads identify an intraparenchymal hematoma. Adapted from Kidwell *et al.* [9].

More invasive approaches are necessary for collecting detailed information at the neuron to neuron level. One novel invasive approach is the stimulated emission depletion microscopy used by Berning *et al.* which makes use of nanoscale resolution imaging techniques to capture information at the single neuron level in mouse cortex [10]. Berning *et al.* used sequential fluorescence to resolve nanoscale features, which are otherwise indiscernible using either confocal or multiphoton microscopy. This study revealed that movements occur in the adult dendritic spines, which was previously unconfirmed. More commonly used techniques involve patch clamp and microelectrode arrays (MEAs), which have also given information about neuron functional activity. Patch clamp uses a glass micropipette to measure ionic currents in individual cells (figure 2.2A), whereas for MEAs, cells sit in contact with or next to single or multiple metallic electrodes that directly measure the changing currents in the area where the electrodes are present (figure 2.2B) [11], [12]. Use

of calcium imaging techniques involve imaging the flux in brightness of fluorescent molecules that respond to the calcium ions through conformational changes modifying their fluorescence (figure 2.2C).

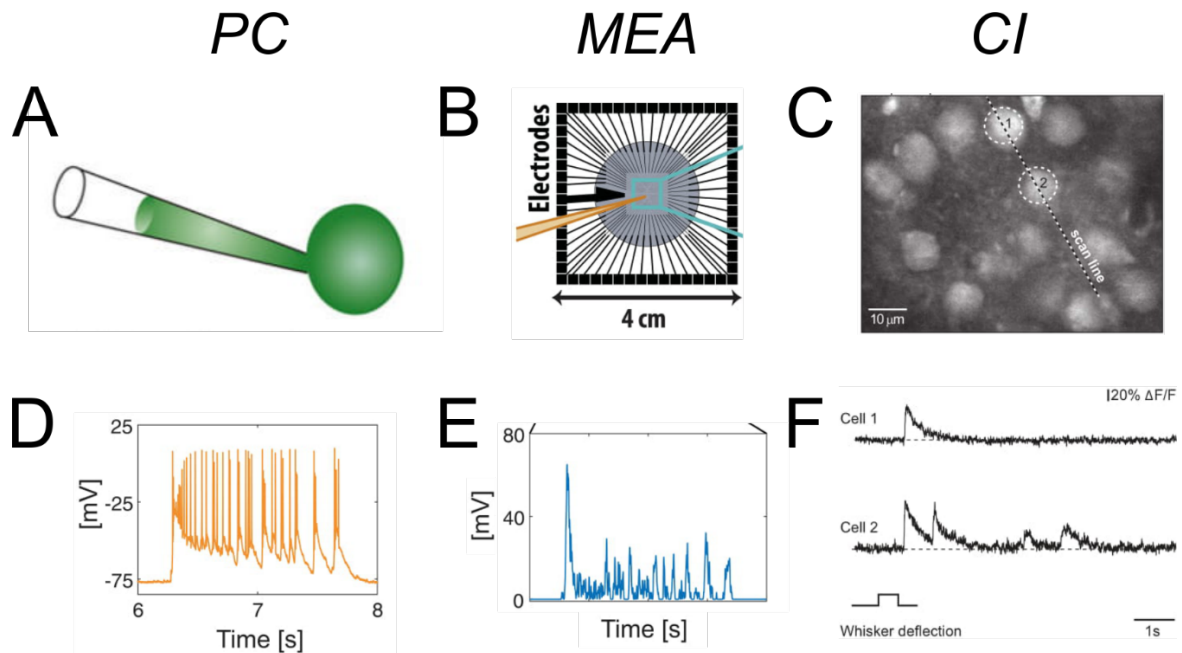


Figure 2.2. Three methods of measuring neural electrical activity. Schematics showing the patch clamp method (PC) (A) and the microelectrode array (MEA) (B). Fluorescent image showing calcium imaging (CI) (C). Graphs of electrical activity vs time for the PC (D) and the MEA (E). Graph showing fluorescence values vs time for CI (F). Adapted from Grienburger et al. and Vardi et al. [13], [14].

Patch clamping gives accurate information of electrical activity within a single cell (figure 2.2D), however it is a difficult protocol to undertake due the requirement of high precision in clamp placement. Moreover, one application of the protocol provides information from only one cell at a time. While MEAs give information about a larger number of cells during each measurement, the necessity of close contact of the cells to the arrays for measurements restricts the number observations that can be collected at one reading using this technique. Calcium imaging techniques are suited to neuronal investigation, because upon

commencement of electrical activity, in response to synapse activation, the current, together with the concentration of calcium ions, rises dramatically in the cell (figure 2.2F) [11], [15]. Therefore, observation of calcium concentration in the cell can give closely related information about the electrical activity of the cell. In terms of neural network functionality assays, calcium imaging provides a valuable characterization approach. In a study by Smetters *et al.*, this method provided information from a much larger sample size when compared with a patch clamp or a microelectrode array approach, and gave signals that were detected without the need for averaging [16]. One drawback of calcium imaging, though, is the slow recovery time as calcium ions diffuse slowly out of the cell, reducing the sensitivity of the technique for detecting individual spikes in spike trains (frequent action potentials). These invasive investigative procedures can be used on human neural networks *in vitro* to provide currently unobtainable information about human neural communication.

2.3. Neurons and Neural Networks

The human nervous system is a complex structure composed of neurons, glial cells, and progenitor cells. Neurons function as the information carrying networks of the system, firing electrical impulses to transmit messages to and from the rest of the body, while glial cells form the support structure of the firing cell network, and progenitor cells line the ventricular space. Neurons consist of a cell body, with extensions that allow them to communicate with each other. These extensions, called neurites, may be singular or branched to facilitate communication with one or many other neurons or other cell types [17], [18].

In the nervous system, neurons are connected in particular networks, to facilitate necessary communication. In the brain cortex, for instance, cortical neurites are aligned lengthwise to facilitate information exchange between layers in the brain (figure 2.3A) [19]–[21]. In the

peripheral nervous system (PNS), axons of neurons are aligned in bundles with other neurons to form nerves [19], [22], [23]. These nerves facilitate communication between the brain and the muscles and sensory organs of the body. Because alignment of neurons plays an important role in complex neural tissue formation, a focus on aligning neurons in the *in vitro* environment is key.

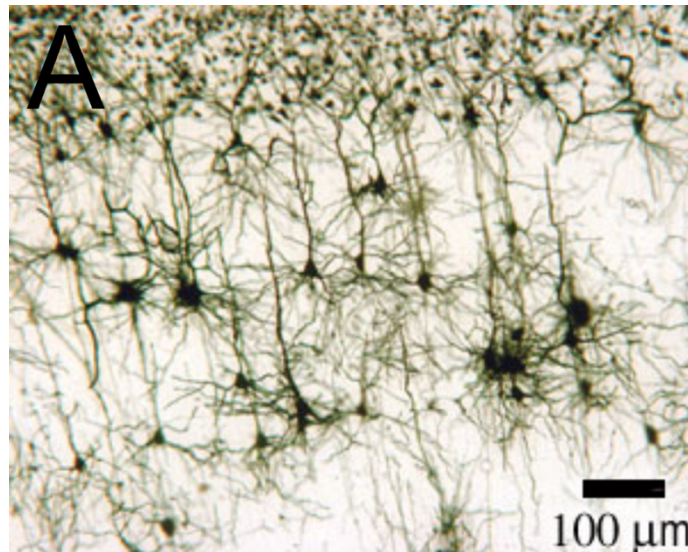


Figure 2.3. A. Image showing a thick section of the cerebral cortex, with neurons stained in black. Adapted from Paxton *et al.* [24].

In vitro, various neuronal models are used including primary cells, cell lines and stem cells. SH-SY5Y cells are human neuroblastoma cells originating from the bone marrow of a 4-year-old female patient. The cells can be treated with all-trans retinoic acid (RA) to induce differentiation into a neuron-like cell stereotype (figure 2.4A, B). This differentiation results in formation of both dendritic and axonal processes bearing the appropriate markers and morphology [25], [26]. In one study, Encinas *et al.* treated the human neuroblastoma cell line with RA and brain-derived neurotrophic factor (BDNF), which resulted in the production of human neuron-like cells [26]. After 5 days in RA culture and 7 days in BDNF culture, not only did the morphology of the neuroblastoma cells resemble that of human

neurons, but also, the cells expressed several neuronal markers that were not expressed in the undifferentiated cells. In another study by Simpson *et al.*, they investigated the effects of retinoic acid and growth factors on nerve growth to determine the difference in effect of these chemicals on the cell line [27]. It was found that while the nerve growth factor (NGF) increased cell proliferation, the RA treatment resulted in increased neurite outgrowth of the cells. SH-SY5Y neuroblastoma cells are hardy, due to their complex genotype, and they are also well established as a neuronal phenotype, expressing mature neural markers once differentiated [28]–[30]. The cells are also easily accessible and widely used in the field for experimental optimization and development [31]–[33]. The SH-SY5Ys were selected as a hardy, representative cell type suitable for this project of developmental nature, with potential to substitute neural induced pluripotent stem cells following optimization of scaffolds.

A number of studies report that SH-SY5Y cells fire action potentials after differentiation with RA [34]–[36]. The cells have also been found to form synapses in the network connections [37]. However, it has not yet been possible to replicate action potential observations using the two available batches of SH-SY5Y cells in the author's laboratory, indicating variability between batches of SH-SY5Ys. For this reason, another neural cell type was needed for full characterization of the scaffolds. F11 cells are from a hybrid mouse neuroblastoma and rat dorsal root ganglion cell line [38]. Protocols to differentiate the cells into neuron-like cells which not only resemble the neurons in terms of neurite extension and network connectivity (figure 2.4C, D), but also in the firing of action potentials, meant that this cell line could be used for characterization of electrical activity within the scaffolds. Differentiation of F11s into neuron like cells involves addition of 8-bromoadenosine 3',5'-cyclic monophosphate (8-br-cAMP), which triggers activation and inhibition of pathways inducing neuronal differentiation [39], [40]. While these cells are non-human, they have

been characterized alongside the human neural cell lines and have been found to express important neural markers, indicating the potential for use in the characterization of the engineered scaffolds [41]–[43]. F11 cells were selected to complement the SH-SY5Y cells and provide important information about the cell activity within the developed scaffolds.

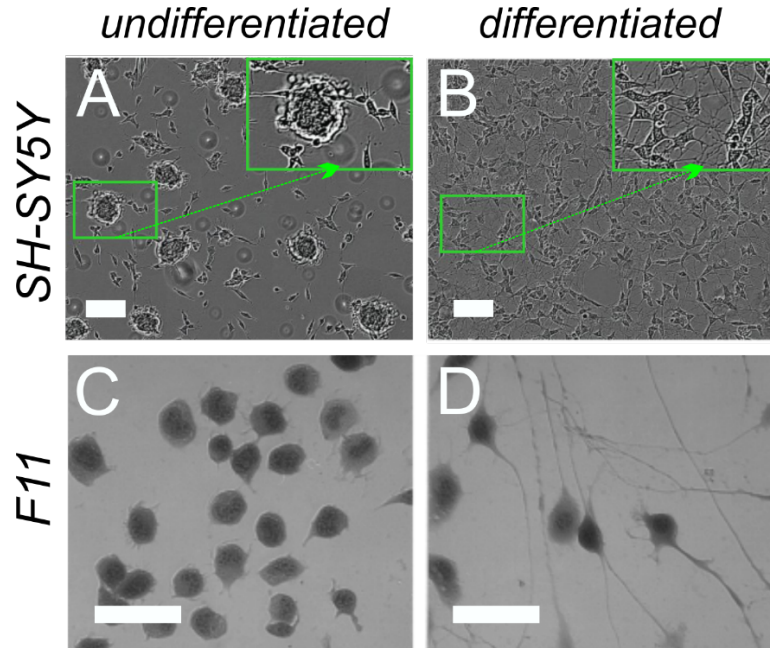


Figure 2.4. Microscopy images showing undifferentiated (A) and differentiated (B) SH-SY5Y cells, and undifferentiated (C) and differentiated (D) F11 cells. For A and B, green boxes show 2X magnification at top right of the images. Scale bars = 100 μm (A, B), 50 μm (C, D). Adapted from Forster et al. and Ghil et al. [33], [40].

2.4. Contact Guidance

One way that the body directs cell growth is through physical surface cues. This is due to cytoskeletal alignment of the cells in a particular direction on the surface in response to the topographical cues. Physical cues are important in the formation of nerves, for instance, where Schwann cells in the nerve assemble themselves into bands of Büngner, which provide topographical cues for the directional growth of axons [44]. In neurons, actin

superstructures called growth cones direct the growth of neurites out from the neuron cell body [45]. Cells are sensitive to their physical surroundings, and often times cells that are proliferating move along shallower angles. In work done by Saito *et al.*, smooth muscle cells seeded on microgroove surfaces grew in the direction of the grooves, and had more mature cell-substrate adhesions when the cells grew in that particular direction [46]. Also, work done by Nemeth *et al.* demonstrated the manipulation of contact guidance in cells [47]. In this study, it was found that not only was the fabricated hydrogel a sufficient cell culture surface, promoting the differentiation of the stem cells, but also that nanopatterning the hydrogel to form microgrooves resulted in directional growth of differentiation of dental pulp stem cells (DPSCs). The approximately 90-degree angles directed cell growth along the grooves rather than perpendicular to them in both studies.

During neurite extension, it is the tip of the growth cone that influences the movement of the entire neurite. According to the cellular tensegrity model proposed by Ingber, the cell is always under a pre-stressed state of tension and compression in order to stabilize structures, and integrin containing focal adhesions sense the mechanical environment and either extend or retract to maintain the cellular stability [48], [49]. The focal adhesions minimize the change of stress by restricting bending movements, and consequently the growth cone and cell is less likely to move along steep topography, and more likely to remain on flat topography. Work done by Dowell-Mesfin *et al.* in 2004 shows that when primary rat hippocampal neuron cells were seeded between pillar grids of varying widths between pillars, the neurites grew in a straight direction whilst being contact guided with narrow enough widths, and were not as guided in a straight direction when the widths between etched pillars were wider, with more flat area available for the cells to explore and proliferate [50].

SH-SY5Y neuroblastoma cells and the hybrid F11 cell line have also been investigated for their reaction to the topographical environment. Tonazzini *et al.* prepared a study using differentiated SH-SY5Y neuroblastoma cells, to investigate the reliability of the cancer cells in representing primary neurons [51]. Using hippocampal neurons as the control, the differentiated neuroblastoma cells were subject to two nanogroove environments and the alignment was measured and compared to the control. The differentiated SH-SY5Ys responded similarly to the primary neurons, leading the authors to conclude that, in fact, the differentiated cell line neurons can be used as a reliable model of neurite outgrowth as the differentiated cells extended long neurites and aligned well with the grooved substrates (figure 2.5D, F). Wieringa *et al.* investigated the potential for alignment of differentiated F11 cells cultured in two different concentrations of fetal bovine serum (FBS) as a metric of robustness for use of this cell line as a neural model [43]. They found that the cells would align themselves along the nanotopographic grooved surfaces for both the 1% (v/v) and 10% (v/v) FBS concentrations, and noted that at the 1% (v/v) FBS concentration, neurites were longer and more aligned.

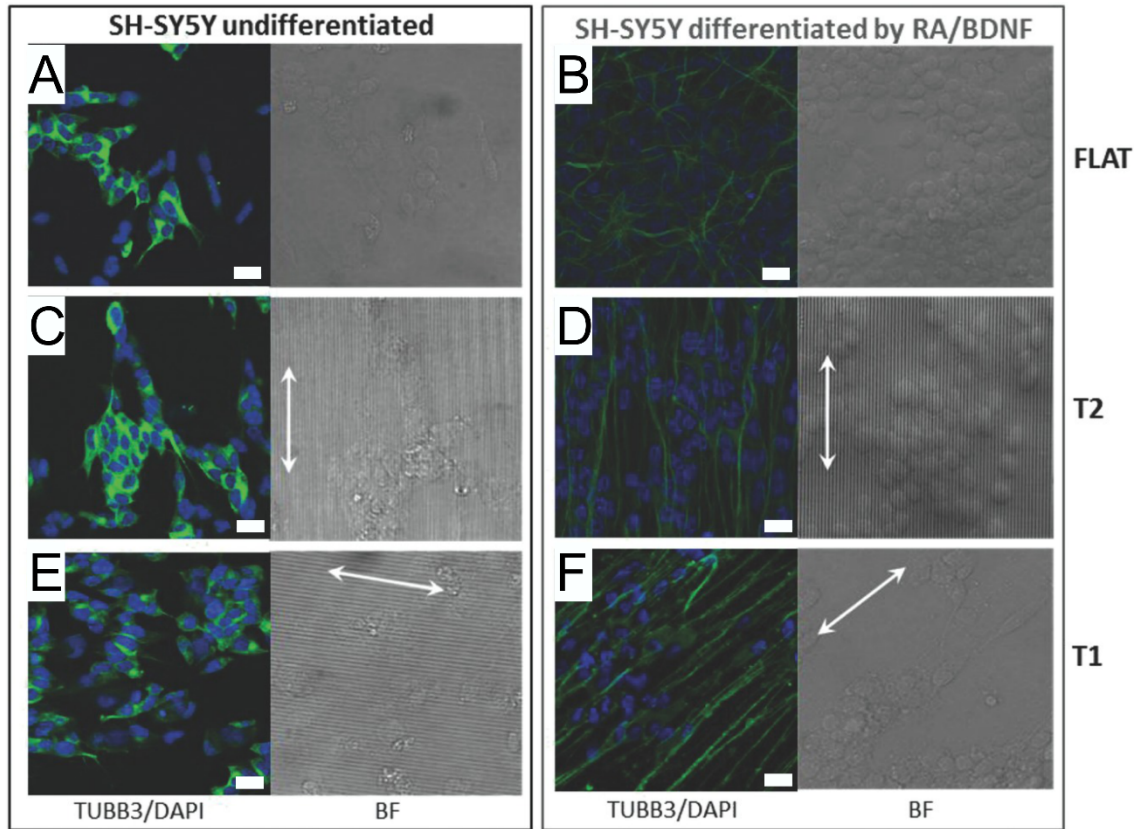


Figure 2.5. Confocal and brightfield images of SH-SY5Y human neuroblastoma cells grown on FLAT (**A, B**), 2 μm groove (T2) (**C, D**), and 1 μm groove (T1) (**E, F**) substrates. The cells were grown for 10 days under standard conditions (left panel, undifferentiated) or neuro-differentiated (right panel, differentiated), and immunofluorescently labelled for Tubulin- β III (green). Nuclei were stained with DAPI (blue). White arrows indicate nanogroove direction. Scale bars = 30 μm . Adapted from Tonazzini et al. [51].

2.5. Surface Patterning Techniques for Cell Culture

A substantial amount of work has been performed to develop cell culture surfaces that create aligned cell networks. Selecting a surface patterning technique involves considering the necessary dimensions, as well as the type of directionality required for the patterned *in vitro* network. A widely implemented surface patterning technique involves the use of polydimethylsiloxane (PDMS) as a non-toxic and biocompatible material that can be

moulded into various shapes using prepared stencils. Choi *et al.* used traditional photolithography to create silicon stencils to form wells in PDMS for the differentiation of neural stem cells [52]. PDMS was also used as the culture surface by Saito *et al.*, who stretched the PDMS sheets to create rounded grooves to investigate the contact guidance effect on rat and bovine smooth muscle cells [46]. It was found that the PDMS material, when coated with gelatin, provided a substantial culture surface, and that the microgrooves that were formed through the stretch and release of the PDMS material affected the orientation of the cells.

PDMS and photolithography techniques have been long used and studied for neural engineering, however alternative fabrication techniques exist. In particular, direct laser machining has been used to harness cellular contact guidance. For instance, Duncan *et al.* demonstrated the direct laser ablation of a polyethylene terephthalate (PET) surface to create grooves for osteoblast cell patterning [53]. Results were similar to those with grooved PDMS material: there was visible cell alignment along the grooves. Moreover, it was found that for the narrower grooves, there was increased cell alignment when compared with grooves of wider spacing. Martinez-Calderon and Ulerich both used one step laser machining processes to pattern stainless steel [54] and titanium [55] surfaces with microgrooves, and found that these topographical patterning improved the control of cell adhesion and migration on these culture surfaces. Studies have also investigated laser machining on electrospun scaffolds. Lee *et al.* used laser machining on a poly (L-lactide) (PLLA) electrospun scaffold to improve the biocompatibility of the scaffold while maintaining the important three-dimensional structure [56]. After the PLLA was heated, charged, and spun onto the collecting drum at various fibre widths, pores were machined in one set of fibres using femtosecond laser ablation at different laser energies, resulting in pore sizes between 50 μm and 200 μm . It was found that human mesenchymal stem cells (MSCs) had higher infiltration in the fibrous

scaffolds with increased pore size. Direct laser machining allows for feature creation on a wide range of material options.

In previous study in the author's laboratory, carried out by Yifan Cai, pores were laser machined in PET membranes at 1 μm , 2 μm , and 3 μm diameter widths [57]. In preliminary seeding tests, it was found that neurites from RA differentiated SH-SY5Y cells extended towards and across the micropores instead of into them. In further investigation on the development of micropores by Matthew Blundell, it was discovered that there was build-up of material in and around the pores, likely caused by melting and deposition of the PET plastic forming blockages within the pores or raised lips around the pores [58]. For this reason, it was deemed necessary to use a material that could be machined to produce pores with smooth entry points and without blockages after machining. The material was changed from a PET membrane to a glass coverslip, and then further investigation was carried out on the porous membrane for cell culture. In Blundell's investigations, pores were laser machined at 90°, 75°, 60°, and 45° angles, measured from the horizontal of the glass. The interaction of seeded neurons with the pores was observed (figure 2.6) and measured, and it was found that, as predicted through the action of contact guidance of neurites, there were more neurons entering into the pores at shallower angles from the horizontal. However, the etch times and acid concentrations used in Blundell's experiments resulted in batch to batch variation in pore widths. In order to optimize this novel porous membrane formation process, it was necessary to further characterize the etch rate and to develop a more reproducible system. The simple ablation of the coverslip by tilting on an angle and laser ablating straight through the tilted glass sample resulted in only one small sector of the pore entry circumference being machined at the desired acute angle. The remaining sectors of the pore entry circumference were machined at angles increasing from this desired angle, until achieving the supplementary angle at the opposite sector. Considering this, it was necessary

to redesign the pore shape into a funnel shape, to facilitate the same desired acute angle at each sector of the circumference, resulting in a multi-directional pore access area on the culture surface.

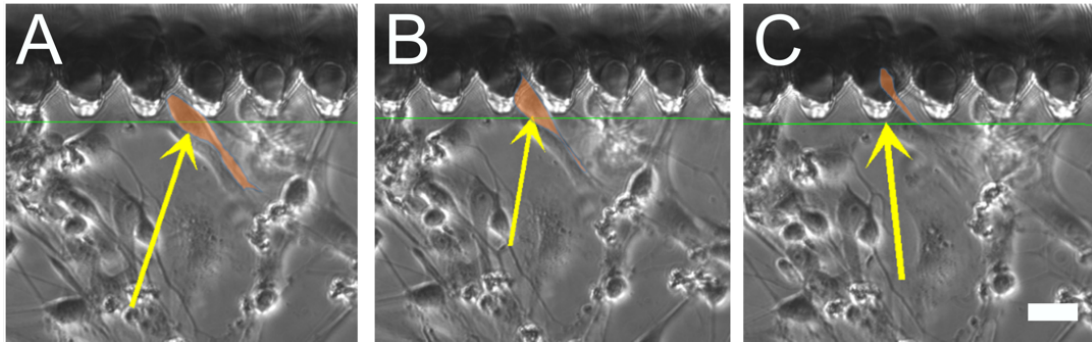


Figure 2.6. Widefield images showing a neuron as it approaches the 60° angled pore. Scale bar = $50\ \mu\text{m}$. Adapted from Blundell [58].

2.6. The Biochemical Environment of the Brain

The extracellular matrix (ECM) is the three-dimensional construct formed mainly from cell-secreted fibrous proteins, glycoproteins, and proteoglycans, that provide structural and biochemical cues for the formation of cells into tissues [59]. The ECM composition guides cell adhesion and arrangement into tissues through protein-protein interactions, where collagen, laminin, and fibronectin in the ECM interact with the integrins and non-integrin receptors in the cell membrane [60], [61]. The ECM also guides cell differentiation and cell secretions through biochemical signalling of transduction pathways in the cell upon adhesion or release from ECM material. In the brain, the ECM is unique in that it lacks abundance of matrix proteins, such as collagen and fibronectin, that are usually present abundantly in the ECM in other parts of the body [62], [63]. Laminin and hyaluronan are important components of the brain ECM [62].

In vitro, to improve the affinity of cells for the artificial scaffold, one or more of these ECM proteins can be incorporated in the scaffold material. The laminin group of glycoproteins, primarily facilitating cell interactions in the basement membrane of the ECM, can be used as a coating on *in vitro* scaffolds to promote adhesive properties for efficient cell seeding [64]. Laminins are heterotrimeric proteins, with three chain variants coded for by 11 genes. The protein chains intersect to form a cross like shape, with three shorter laminin-binding arms at the N-terminus, and one longer cell-binding arm at the C-terminus [65]. Laminin in the ECM binds to cells through protein-protein interactions between residues in the C-terminus of the laminin chain and integrins in the cell membrane. Of the 15 laminins, laminin-111 was selected for this project as it is well studied and established, and also because it is the variant involved in neural development, promoting axonal growth [66].

Once cells are adhered in the scaffold, cell growth can be directed in the body through setting up chemotaxis gradients. As with the physical environment, cells are very sensitive to their chemical environment, and therefore chemical binding cues are useful in cell patterning. Studies investigating the phenomenon of chemotaxis *in vitro* have shown that a gradient of growth factors influences cell growth in the direction of higher concentration. Del Amo *et al.* studied the growth and distribution of fibroblasts embedded within a collagen hydrogel, set up in a microfluidic device, and found that the gradient of platelet derived growth factor and vascular endothelial growth factor (VEGF) directed the cells to move towards the increased concentration of growth factors in the device [67]. However, it was interesting to discover that for very high concentration gradients, the effect was not seen as much as for lower concentration gradients. This could be explained by the increased diffusion of growth factors across the investigated microfluidic space, diminishing the effect of the gradient. Similarly, Fiorini *et al.* used chemotaxis to regulate the migration of mesenchymal stem cells into the polyamidoamine based hydrogel [68]. Moreover, the stromal cell derived factor 1 α

chemoattractant used in this study was also found to reduce the inflammatory effect upon implantation of the scaffold for *in vivo* animal testing, optimizing the chemoattractant functionalized scaffold over the non-functionalized hydrogel scaffold.

In neurons, the growth cone directs cell movement along chemical gradients by sensing the gradient, and regulating the cytoskeletal motility in the direction of that gradient [69]. Reelin is a chemoattractive extracellular protein expressed by Cajal-Retzius cells present in the developmental cerebral cortex, where it forms a linear concentration gradient. This directs the growth cones towards linear alignment within and between brain layers [70]. BDNF is responsible for growth, differentiation, and maintenance of the neurons in the CNS and PNS, and *in vitro*, gradients of BDNF as well as other growth factors have been used, including NGF, VEGF, and netrins [71]–[73]. A study involving BDNF and VEGF seeded into hyaluronic acid hydrogels showed improved growth of neural stem cells compared to the control [74], and another study involving the same factors added to silk fibroin scaffolds showed accelerated Schwann cell growth compared to the control without BDNF and VEGF [75]. The incorporation of BDNF into scaffolds has also improved the neurite growth and directed innervation of regenerating axons in rat injury models. In a study by Jain *et al.*, agarose hydrogels were embedded with BDNF and injected into spinal cords of rats after a portion of the cord was surgically removed [22]. There was improved neurite growth into the scaffolds and also a reduction of the inflammatory effect for the BDNF impregnated agarose hydrogels compared with the non-BDNF agarose hydrogels.

2.7. Three-Dimensional Cell Culture Techniques

It is important to consider the cell culture environment in creating a representative *in vitro* engineered tissue. Ideally, the environment should replicate aspects of the *in vivo* environment, having tailored stiffness, and allowing cell migration and diffusion. Typical adherent cell culture involves cell growth on flat plastic, glass, or similar surfaces. This type of cell culture is useful for many types of cell investigation, and many observational and analytical techniques have been optimized for use on two-dimensional (2D) culture. In the *in vivo* environment, the cells interact with other cells, the extracellular environment (ECM) and the surrounding chemicals, experiencing mechanical and chemical cues not only along a 2D surface, but in all three dimensions of the ECM. Cells that are cultured on two-dimensional surfaces are morphologically and phenotypically different from cells that are exposed to the three-dimensional (3D) *in vivo* environment. 3D scaffolds allow cell culture with appropriate external inputs that result in the development of complex cell networks, that function as a more representative model for native cell networks [4], [76].

Hydrogels are materials with high water content that have proved to be a good representative for the 3D culture of soft tissues. According to Liu *et al.*, hydrogels work well in soft tissue engineering due to biocompatibility and ease of manufacturing the hydrogel to allow sufficient oxygen and nutrient exchange, and according to Nih *et al.*, hydrogels can provide structural and biochemical support for *in vivo* applications [77], [78]. Frequently used natural hydrogels include hyaluronic acid, collagen, and gelatin [79], [80]. However, it is important to consider the mechanical environment as this will affect the ability of cells to grow and proliferate adequately in the scaffold. In a study concerned with the culture of neurons from human chorion-derived MSCs, Faghihi *et al.* cultured the cells on an electrospun and crosslinked gelatin scaffold [81]. Positive expression of neural specific genes indicated successful differentiation of the cells in the three-dimensional scaffold. Lou

et al. cultured MSCs in a composite hyaluronic acid and collagen hydrogel scaffold, with novel crosslinking methods to improve the relaxation dynamics in the scaffold [82]. This resulted in improved formation of focal adhesions and cell spreading in the scaffold compared with conventional hydrogels prepared with static crosslinking methods.

Synthetic hydrogel models have also become frequently used for cell culture. These hydrogels, made from the crosslinking of polymers such as polyethylene glycol (PEG) and polyglycerol sebacate (PGS) have some advantages over the natural hydrogels. Apart from having reproducible physical and chemical properties, synthetic hydrogels are more mechanically stable in long term cultures. For *in vivo* applications, they form scaffolds that have lower potential to produce immunogenic responses [78]. Photodegradable PEG hydrogels were used to control cell migration in a study by Kloxin *et al.*, where real time degradation of the synthetic hydrogel resulted in defined areas into which cells were directed to migrate [83]. Bettinger *et al.* used PGS to prepare multi-layer hydrogel structure using microsheets that increased the relative surface area for cell attachment of hepatocytes (figure 2.7) [84]. Cellular attachment and high albumin production indicated long term cell viability and liver-specific functionality of the cultures.

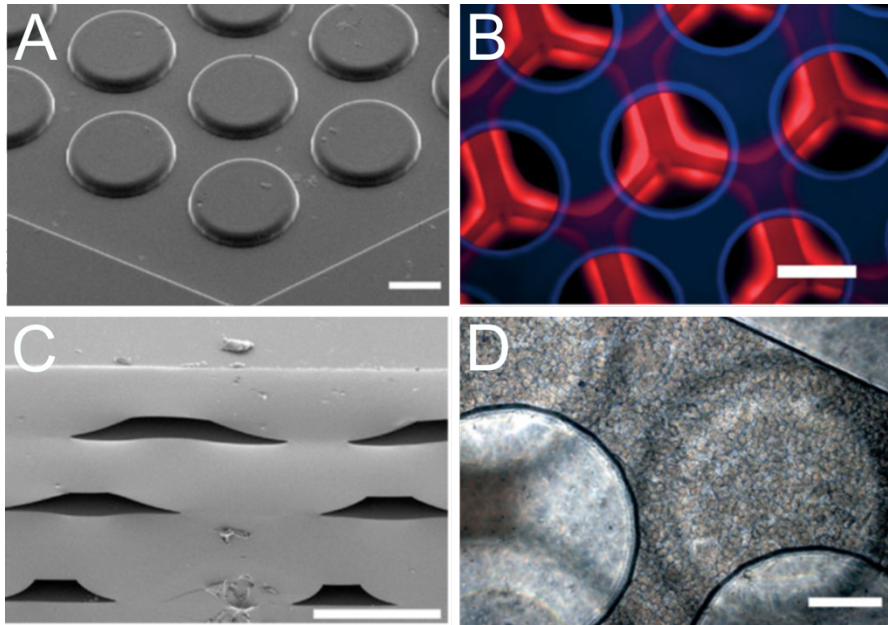


Figure 2.7. Multi-layer hydrogel structure for hepatocyte culture. **A.** Moulded PGS layer. **B.** Composite fluorescent micrographs of the device, showing multi-layer nature. **C.** SEM of cross-section of the three-layer scaffold. **D.** Brightfield images showing confluent hepatocyte cultures. Scale bars = 200 μm . Adapted from Bettinger et al. [84].

While synthetic hydrogels have some benefits over the natural counterparts, the biocompatibility and cellular affinity of the scaffold is important, and often cannot be sacrificed. Therefore, it has become common to use combination scaffolds, which apply synthetic modifications to natural hydrogels, to harness the advantages of both types of hydrogels, and more importantly, to maintain the biocompatibility of the scaffold. Hyaluronic acid, for instance, can be conjugated with functional groups such as thiols, amines, or acrylates, to form a hydrogel which can be reproducibly customized for chain length [85]–[87]. The functionalized acid is cross-linked with a reactive crosslinking agent, which allows the user to prepare the hydrogel for each use. This gives the option to encapsulate cells and various growth factors into the hydrogel before crosslinking.

The synthetic hyaluronic acid hydrogel chemical process involves first functionalizing the disaccharide polymer with a thiol group (figure 2.8A). One of the carboxyl groups on the saccharide backbone of the hyaluronan is replaced with an amide containing the thiol group using a carbodiimide-activation [88]. The thiol groups can spontaneously form disulphide bonds in air (figure 2.8B), but this crosslinking process is slow, and addition of a PEG-diacrylate component can speed up the crosslinking process at physiological pH and temperature, via Michael-type addition between the thiol group on the functionalized hyaluronan and the acrylate group on the PEG-diacrylate (figure 2.8C) [89].

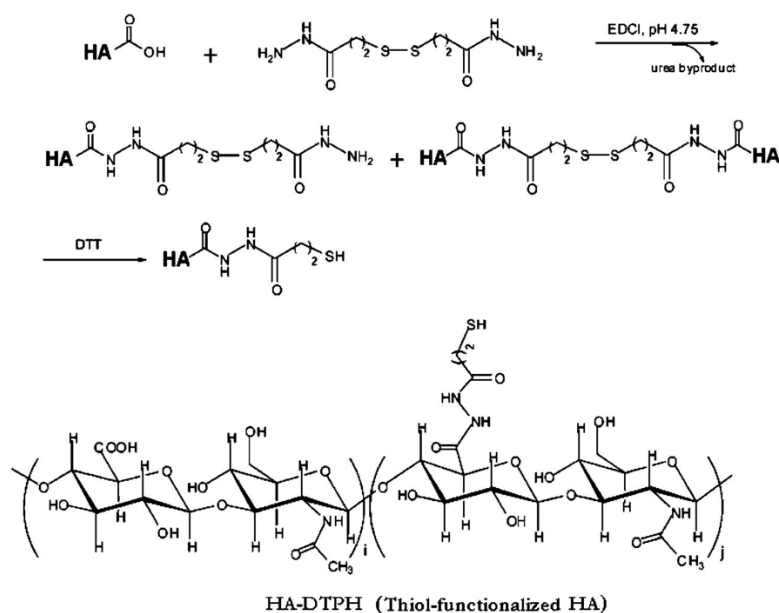
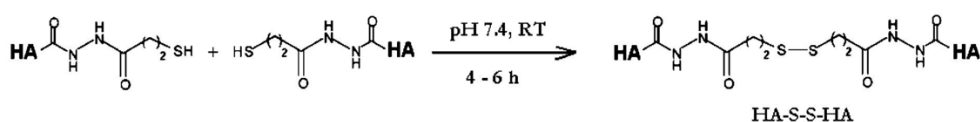
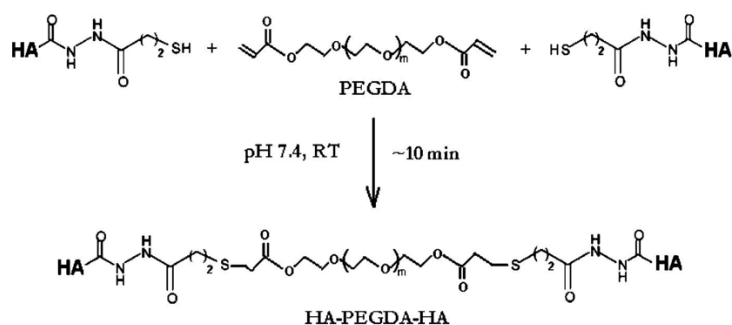
A**B****C**

Figure 2.8. Schematic showing (A) synthesis of thiol-functionalized HA (HA-DTPH), (B) spontaneous cross-linking forming HA-S-S-HA linkages, and (C) exogenous PEGDA-mediated cross-linking forming HA-PEGDA-HA linkages. Adapted from Ghosh *et al* [89].

Nemeth *et al.* used similar functionalized hyaluronan hydrogel, with a modified gelatin construct as well as a PEG construct, to study the migration differentiation of DPSCs [47]. The hydrogel was combined, patterned with nanometre grooves, and the DPSCs were cultured on the nanopatterned material for several days. The study showed that the hydrogel material provided a surface that could efficiently culture cells and promote the

differentiation of DPSCs. In a previous study in the author's laboratory, Phillipus Putter synthesized and characterized several hyaluronic acid based hydrogels, investigating the effect of the hydrogel environment on cell growth [90]. Putter modified the hyaluronic acid hydrogel by adding chemicals such as thiol, to result in predictable crosslinking, and by changing the concentration of the chemicals and cross linker, to result in predictable stiffness modifications. NTera2.D1 cell-line cells were encapsulated in a hyaluronic acid hydrogel with laminin, resulting in the growth of a dense cell network, indicating that the material was sufficient to support a three-dimensional neural cell culture (figure 2.9C, D). Unfortunately, while there was the increased neurite outgrowth with time in the hydrogels, it was also observed that the neural networks formed were discrete clusters of rounded cells. Native neural cultures exist as connected networks with elongated, non-rounded cells. It is therefore necessary to make improvements in this hydrogel scaffold, providing a 3D environment facilitating formation neural cultures more representative of native neural cultures.

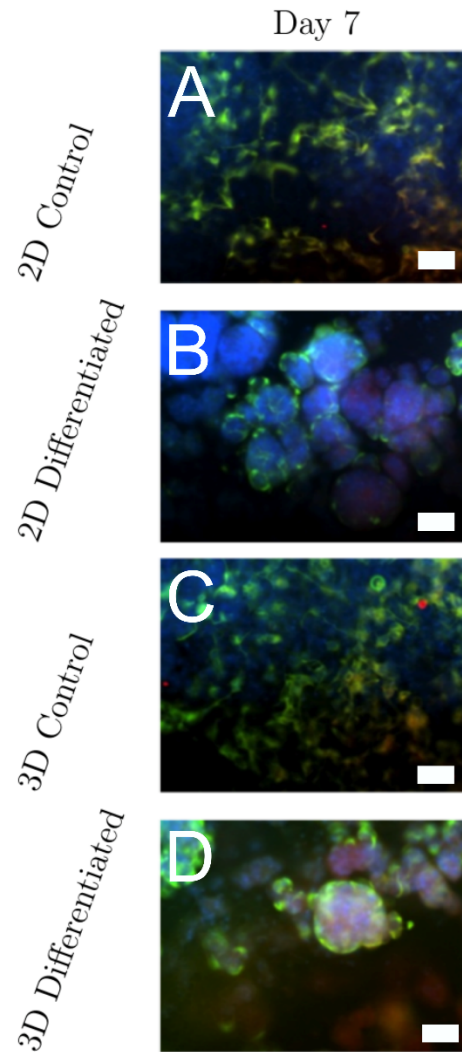


Figure 2.9. Encapsulation and differentiation of Ntera2.D1 cells. Undifferentiated (**A**) and differentiated (**B**) cells were seeded on tissue culture plastic as a 2D control to compare with encapsulation of undifferentiated (**C**) and differentiated (**D**) cells in the HA hydrogel. Cells were stained for tubulin (green) GFAP (red) and DNA (blue). Scale bars = 100 μm . Adapted from Putter [90].

2.8. Peripheral Nerve Damage and Regeneration

The *in vivo* process of nerve regeneration after a nerve is severed, as described by Ron *et al.*, involves initial swelling, followed by the clearing away of damaged tissue by dedifferentiated Schwann cells and recruited macrophages (figure 2.10B, C) [91]. This process, termed ‘Wallerian degeneration’, results in disintegration of the cytoskeleton and removal of myelin debris, and allows the damaged regions of the nerve to be cleared and prepared for the newly regenerating tissue. After the degeneration process, the Schwann cells assemble themselves into bands of Büngner by proliferating along the remainder of the endoneurial tubes which formed part of the extracellular matrix in the nerve (figure 2.10D). The proliferating Schwann cells form an aligned structure that provides topographical cues for the directional regeneration of axons. The Schwann cells also secrete neurotrophins NGF and BDNF which provide chemoattractive cues for the axons as well as neurite promoting factors of fibronectin and laminin which increase neurite extension across the gap. Regeneration in the nerve then commences with the sprouting of a growth cone from the proximal stump of the damaged nerve (figure 2.10D). This growth cone explores the environment and experiences both the topographical and chemotaxis cues provided by the Schwann cells. Over time, the axon grows towards the distal stump, eventually reconnecting with and reinnervating the stump, provided the gap is not too wide (< 4 mm), and that there are no blockages from scar tissue preventing connections from being made (figure 2.10E).

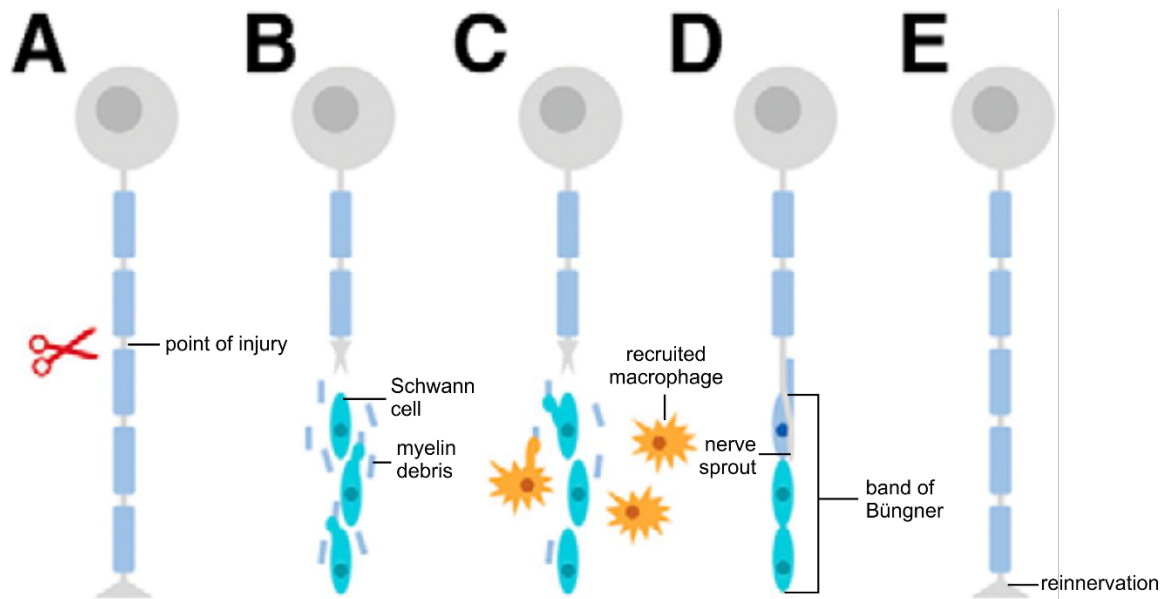


Figure 2.10. Schematics showing nerve injury and regeneration. At the point of injury (A), Schwann cells commence recruitment of macrophages and removal of myelin debris, also known as the Wallerian degeneration process (B, C). Schwann cells then form bands of Büngner (D) and nerve regeneration and the nerve sprout grows to innervate the stump (D, E). Adapted from Fernandez *et al.* [92].

There are times when the natural regeneration process fails, because damage is severe and the gap is too wide, and in these cases, medical intervention is necessary. According to Chiono *et al.*, the preferred medical approach is nerve coaptation: suturing the proximal and distal ends of the damaged nerve together, which improves the chance of reinnervation by reducing the distance between the stumps and also preventing any further physical blockages from coming between the stumps [93]. Moreover, when the stumps are coapted using surgical procedures, the regeneration process can proceed without the infiltration of fibroblasts in a foreign body response. However, if the nerve gap is too wide (> 4 mm), the stumps cannot be coapted without applying tension, and the suturing operation results in constriction of the nerve at the suture location, preventing reinnervation [94]. In these cases, an autograft can be used for bridging the gap and connecting the damaged nerve stumps, but

issues of size and morbidity arise with such an option. A harvest surgery is necessary to collect the autograft, and there is a potential issue if the collected autograft is a misfit in terms of length and/or diameter. It is of great interest to design artificial grafts that can be prepared with the desired dimensional specifications and nerve guidance cues, to replace autografts and be implemented without the need for additional surgery.

2.9. Artificial Nerve Graft Conduit Technology

Artificial nerve graft conduits (NGCs) have been developed and investigated for use in wider nerve gaps. The current medically approved NGCs range from simple non-porous, hollow tubes, to semipermeable, hollow, mesh tubes, which can be sutured to the nerve stump ends (figure 2.11) [6], [95]. These grafts are fabricated from both natural and artificial polymers such as collagen, porcine small intestinal submucosa, PGA, polycaprolactone (PCL), and silicone, and they restrict fibroblast infiltration and therefore allow regeneration to occur in an isolated space. They also maintain the flow of neurotrophic factors that are being secreted by the nerve cells. However, they do not mimic the topographical or chemoattractive cues provided *in vivo* and for this reason do not work for very large nerve gaps, that is, gaps greater than 30 mm.

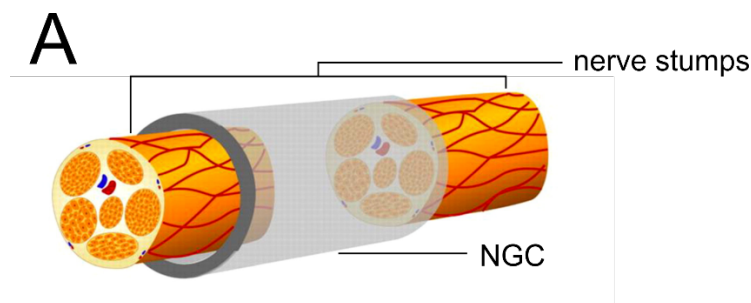


Figure 2.11. A. Schematic showing the current medically approved artificial nerve graft conduits – consisting of hollow, non-porous or semipermeable tubes. Adapted from Chiono *et al.* [93].

Current research investigates production of more complex nerve conduits, which either add topographic cues to the inner portion of the tube wall, include neurotrophic factors within the NGC, or include fillers within the lumen of the NGC. Mobasseri *et al.*, for example, investigated different groove designs on PCL and PLA films formed into 14 mm conduits [96]. *In vitro*, it was found that adipose derived Schwann-like cells significantly improved cell attachment and alignment in the grooved versions of the conduit compared with the non-grooved versions. *In vivo*, regenerating nerves in the grooved conduits maintained a greater area than those in non-grooved counterparts. Poly-L-lysine was used in another study to functionalize PCL melt extruded nerve guides, and this improved the cell adhesion and cell density of neuroblastoma cells in the conduits, compared with unfunctionalized PCL conduits [97].

Concerning the use of conduit fillers, in a study by Nakayama *et al.*, copolymer conduits filled with fibrin hydrogel were implanted as nerve grafts in rats, and the total area of myelinated nerves within the tube 12 weeks after implantation was measured and compared with the same in unfilled conduits [98]. It was found that the fibrin filled conduits allowed regeneration encompassing a greater area than in the unfilled conduits. Internal gelatin sponge fillers within a PCL nerve guide was also shown to improve adhesion and proliferation of Schwann cells in a similar study [99].

Some studies have opted to add a combination of chemoattractive cues to the conduit design. A microgroove modified substrate was investigated by Miller *et al.* and it was found that the combination of physical and chemical cues improved the neural regeneration [100]. Poly-D, L-lactic acid and PLGA films were prepared, and an imprinting method was used to pattern microgrooves onto the scaffolds using quartz and silicon patterned substrates. Laminin was selectively adhered to the microgroove edges and dorsal root ganglion (DRG) were dissociated and seeded onto smooth, microgrooved, and laminin adhered

microgrooved, substrates. While there was longer neurite extension on the smooth substrate compared with the microgrooved substrate, the alignment of the neurites was improved with the microgrooves. Furthermore, the adhesion of laminin to the substrates improved neurite outgrowth as well as neurite adhesion.

Similarly, Jenkins *et al.* added multiple cues to the NGC design [101]. The team added topographical cues by preparing an electrospun PCL conduit. The PCL fibres were electrospun around drawn sucrose fibres to form an aligned fibrous sheet which was then rolled into a conduit shape. Following dissolution of the sucrose, a PCL conduit with hollow aligned microchannels was formed. The team also added biochemical cues by preparing some composite conduits with PCL, polyserinol hexamethylene urea and arginylglycylaspartic acid (RGD) groups. Upon seeding with human neural stem cells, the NGCs with both topographical and biochemical cues showed improved neurite growth and extension compared with pure PCL conduits. Notably, the composite conduit had a higher cell density than the PCL conduit, indicating that a combination of cues improved the function of the NGC. However, the investigation found that neurite growth extended over only 15 mm, a much shorter distance than is possible using current medically approved NGCs.

These modifications to the hollow tube bring the artificial NGC design closer to mimicking the *in vivo* nerve, and therefore such NGCs are expected to be more successful in regeneration of these wide nerve gaps than the simple hollow NGC designs. However, the increased complexity of the NGC usually involves a more complicated fabrication process as well, as seen with the multi-step electrospinning process as investigated by Jenkins *et al.*, or with the specific imprinting process investigated by Miller *et al.* The straightforward hydrogel fabrication and seeding process investigated in this thesis showed potential for further application, which motivated the decision to work towards preparing a device that

produces tertiary structure suitable for improved NGC technology for PNS regeneration, as well as for complex CNS models.

2.10. Summary

Investigations into material manipulations for the purposes of improved control of cell growth, have directed the experimental plans examined in this thesis. The phenomenon of contact guidance led to the hypothesis that shallow angled pore entry could be used to direct and isolate the neurites of low-density neural cultures. A desire to further investigate the hyaluronic acid hydrogel scaffold inspired the idea to modify the mechanical environment to enhance neurite extension and network connectivity of three-dimensional neural cultures. Finally, potential for application of the modified hydrogel towards improved NGC technology motivated the development of a device to add tertiary structure to the hydrogel, making it more representative of the native ordered scaffold structures of the PNS and the CNS. These distinct investigations share the same goal of gaining control over scaffold materials to direct neural network formation, with the intention of increasing knowledge in the field, developing representative complex *in vitro* models, and advancing medical technologies.

Chapter 3: Materials and Methods

3.1. Cell Culture

3.1.1. *SH-SY5Y Human Neuroblastoma Cell Line*

The SH-SY5Y neuroblastoma cell line (ATCC, UK) was cultured in 75 cm² tissue culture plastic (TCP) flasks in complete medium composed of RPMI 1640 with stable Glutamine (PAA Laboratories, UK), supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, UK), 100 U/ml penicillin with 100 mg/ml streptomycin (1% (v/v) P/S, PAA Laboratories, UK) and 0.1 mM non-essential amino acids (1% (v/v) NEAA, PAA Laboratories, UK). Culture medium was exchanged every 2 days until cells reached 70% confluency. The cells were then differentiated for 6 days with the addition of RA (Sigma Aldrich, UK) by preparing a 10 μ M RA solution in complete medium supplemented as previously described, exchanging medium every 2 days. At the end of the differentiation process, RA differentiated SH-SY5Y cells were used for further experimentation.

3.1.2. *F11 Rat Hybrid Neuroblastoma and DRG Cell Line*

The F11 neuroblastoma cell line (ECACC, UK) was cultured in 75 cm² tissue culture plastic flasks in complete medium composed of DMEM, high glucose (Gibco, UK), supplemented with 10% (v/v) FBS and 1% (v/v) P/S. Culture medium was exchanged every 3 days until the cells reached 95% confluency. Cells were then used for further experimentation, differentiating them after cell seeding.

3.2. Cell Seeding of Polycarbonate Microporous Membranes

3.2.1. Polycarbonate Membrane Preparation

Polycarbonate track etched membranes (Millipore, UK) with 1.2 μm pores and with 8 μm pores, were sterilized, oxygen plasma treated, and coated with laminin (Laminin-111, molecular weight 900 kDa, Sigma Aldrich, UK) at an equivalent concentration of 2.5 $\mu\text{g}/\text{cm}^2$ in 500 μl phosphate buffered saline (PBS, Sigma Aldrich, UK) per 24 well plate well. Samples were incubated at 37°C for one hour.

3.2.2. Cell Seeding for Quantitative Analysis

RA treated SH-SY5Y neuroblastoma cells were trypsinized and centrifuged, and the cell pellet was re-suspended at (0.33, 0.67, 1.00, 1.33, 1.67, and 2.00) $\times 10^6$ cells/ml. Cells were seeded onto the 1.2 μm laminin coated track-etched membranes at 30 μl per 8 mm diameter mini-well, created by inserting PDMS O-rings into the well, resulting in seeding densities of (2, 4, 6, 8, 10, and 12) $\times 10^4$ cells/ cm^2 . Samples were prepared in triplicate and maintained at 37°C for one week with media replenished every other day before fixing in 4% (v/v) paraformaldehyde. Samples were stained with rhodamine phalloidin tetramethylrhodamine (TRITC) (Sigma Aldrich, UK) and the underside was imaged using confocal microscopy. Images were analysed using ImageJ software (NIH, USA) to calculate percentage fluorescence coverage over 3 images per membrane of the underside area. Statistical analysis was carried out on the whole dataset using ANOVA single factor test, with $p < 0.05$ as the total threshold for statistical significance. Three Bonferroni corrected post-hoc student's t-tests were then used to analyse further differences between groups, with $p < 0.017$ as the adjusted threshold for statistical significance.

3.2.3. *Cell Seeding for Qualitative Analysis*

Cells were seeded in a similar fashion onto three 8 μm coated polycarbonate membranes at a seeding density of 1×10^4 cells/ cm^2 . Cultures were maintained for 24 hours before capturing images of cell exploration using high magnification imaging with a widefield fluorescence microscope, set up to capture differential interference contrast (DIC) microscopy (Nikon, UK). Images were captured every minute for 4 hours and images were observed in order to select areas of interest using ImageJ software. Videos of 4 cells interacting with the pore edges were analysed qualitatively.

3.3. **Development of Funnel-Shaped Micropores on Glass Coverslips**

3.3.1. *Glass Coverslip Laser Machining*

Laser machining was used to create a set of funnel-shaped pores (supplementary figure S1). These pores were fabricated by a collaborating laboratory, the Dynamic Optics and Photonics group at the Department of Engineering Science, University of Oxford. Square glass coverslips were ablated using a regeneratively amplified titanium sapphire laser (Newport Spectra Physics Solstice, UK). The machining was completed at room temperature, in open air. Attenuation of the pulses was achieved using a rotatable half-wave plate and a Glan-Laser polarizer. Following this, the beam was placed in the path of a reflective liquid crystal phase-only spatial light modulator [102]. The glass coverslips were mounted onto a three-axis air-bearing translation stage.

On each square glass coverslip of thickness 0.17 mm (Fisher Scientific, UK), a grid of nine circular funnel-shaped pores was ablated. The funnels were ablated at 60 μm in diameter at the mouth, and 1 μm at the stem, with 100 μm separation between funnels, mouth edge to mouth edge. Funnel angles were machined in three groups: shallow, moderate, and steep.

Samples for etch time characterization were prepared with all three groups on one coverslip, while experimental samples were prepared with each angle group on a separate coverslip.

3.3.2. Glass Coverslip Etch Time Optimization

An 80 ml volume of 1% (v/v) hydrofluoric acid (HF, Sigma Aldrich, UK) solution was made by slowly adding 1.6 ml of 50% (v/v) HF acid to 78.4 ml deionized water. The laser machined coverslips were placed in 50 ml conical tubes (Corning, UK) and the tubes were placed into a sonicator (Crest Ultrasonics, USA). A volume of 10 ml of 1% (v/v) acid solution was added to each tube to cover the glass coverslips, and then etching was allowed to occur for 10, 20, 30, 40, 50, 60, 75, and 90 minutes. The acid solution was removed from the tubes after the time points and placed into to a plastic beaker containing 2M calcium carbonate neutralizing buffer. The glass coverslips were rinsed in deionized water, then in ethanol (Sigma Aldrich, UK), to remove any remaining HF acid solution and cease the etching process. The slides were allowed to dry in the fume hood. Results were used to advise modifications in the laser machining and etching process.

After optimization, HF etching was allowed to occur for 40 minutes, 45 minutes, and 50 minutes respectively for the shallow, moderate, and steep funnel-shaped pore samples.

3.3.3. Glass Coverslip Annealing

Glass coverslip samples were annealed as a method of reducing the surface roughness of the micropore features on the glass coverslip, as heating and cooling glass at specific temperature gradients has been shown to relieve the internal stresses and reduce the surface roughness [103]–[105]. Samples from both angle groups were placed into a furnace (Thermo Fisher Scientific, UK) and the temperature was allowed to increase at a rate of 13°C/min. The first set of samples was allowed to heat at 500°C for one hour. A second set of samples

was annealed at 600°C for two hours. Experimental samples were heated at 600°C for 3 hours. After the specified amount of time, the furnace was allowed to cool to room temperature at a rate of approximately 2°C/min, and then the samples were carefully removed.

3.3.4. Glass Coverslip Cell Seeding

The square glass coverslips each with 9 funnel-shaped pores were exposed to a 70% (v/v) ethanol solution in deionized water for 30 minutes at room temperature. The coverslips were then autoclaved and placed into a petri dish, after which they were placed into an oxygen plasma chamber (Diener Electronic, Germany) and treated at 160 W and 20 Pa for 30 seconds, before being soaked in a sterile solution of 2.5 µg/cm² equivalent concentration of laminin in PBS, in a tightly covered petri dish, for one hour in an incubator (37°C, 5% (v/v) CO₂). In a sterile tissue culture hood, green fluorescent protein (GFP) modified RA differentiated SH-SY5Y cells (Aston University, UK) were harvested using trypsin (Sigma Aldrich, UK). Cells were seeded onto the coverslips at 5 x 10⁴ cells/cm², and the coverslips were incubated for 30 minutes to allow attachment of cells. After attachment, cells were allowed to proliferate in neuronal base medium (NBM, PAA Laboratories, UK) supplemented with L-Glutamine (PAA Laboratories, UK), 2% (v/v) neuronal stem cell supplement (PAA Laboratories, UK), 1% (v/v) P/S, 1% (v/v) NEAA, 10 ng/ml (0.38 nM) NGF (Sigma Aldrich, UK) and 50 ng/ml (3.6 nM) BDNF (PeproTech, UK). Medium was exchanged every 2 days for a total of 4 days. The experiment was repeated to collect 2 independent datasets for analysis.

3.3.5. Image Collection and Analysis

The unseeded glass coverslips were imaged using a profilometer (Alicona, UK). Short focus optical images were taken through the entire vertical distance of the funnel. Using the Alicona profilometer, profiles of the funnels were taken as well as surface roughness measurements.

Acellular samples were also imaged using a scanning electron microscope (Zeiss, UK). Coverslips were sputter-coated in platinum and then imaged flat on the horizontal under high vacuum conditions to capture high resolution images for qualitative analysis.

For cell seeded samples, live cell imaging was carried out using a widefield fluorescence microscope (Nikon, UK). Membranes were placed in an enclosed heated and gassed microscope stage top chamber (37°C, 5% (v/v) CO₂, Okolab, USA), and then imaged using brightfield and fluorescence imaging using a FITC filter set at 20X magnification, every minute for 120 minutes. The number of neurons crossing the funnel boundaries in the field of view was measured for the different angled funnel-shaped features at 13 selected time points within the full dataset: $t = 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110,$ and 120 minutes.

Image processing software, ImageJ, was used to analyse the collected images. Angle and diameter dimensions of the resulting funnel and pore features were measured. Live cell data was statistically analysed using ANOVA single factor test across the whole dataset, with $p < 0.05$ selected as the threshold for statistical significance. This was followed by four Bonferroni corrected post-hoc student's t-tests to analyse differences between groups, where the threshold for significant difference was selected at $p < 0.013$.

3.4. Development of Granular Hyaluronic Acid Hydrogels

3.4.1. Hyaluronic Acid Hydrogel Fabrication

Using a syringe (BD Plastipak, UK) and aseptic technique, a hyaluronic acid hydrogel (HyStem kit, Sigma Aldrich, UK) was prepared. The hyaluronic acid backbone was pre-functionalized with a thiol group via a carbodiimide activation, dehydrated, and the kit was maintained at -20°C until it was time to prepare the hydrogel. A 1% (w/v) hydrogel was prepared according to fabrication instructions as follows: dehydrated thiol-modified hyaluronic acid hydrogel was reconstituted with 1 ml degassed water, and the thiol-reactive PEG-diacrylate crosslinking agent was reconstituted with 0.5 ml degassed water. A 2% (w/v) hydrogel was prepared as follows: dehydrated thiol-modified hyaluronic acid hydrogel was reconstituted at double concentration, with 0.5 ml degassed water, and the thiol-reactive PEG-diacrylate crosslinking agent was reconstituted according to the fabrication instructions with 0.5 ml degassed water. Reconstituted hyaluronic acid was allowed to mix on a roller for one hour before combining with the crosslinking agent.

The 1 ml volume of 1% (w/v) hydrogel was combined with 0.25 ml crosslinker and the 0.5 ml volume of 2% (w/v) hydrogel was combined with 0.25 ml crosslinker. To prepare the non-granular hydrogel, the combined hydrogel and crosslinking agent were allowed to crosslink via Michael-type addition, at 37° C for 24 hours. To prepare the granular hydrogel, the non-granular hydrogel of the same concentration (1% or 2% w/v) was granulated by pressing through a 40 µm cell filter (Corning, UK), a 17 µm transmission electron microscopy (TEM) grid or a 13 µm TEM grid (Agar Scientific, UK). When cells were added to the hydrogels, the granular hydrogels were crosslinked and granulated before cells were added along with a post-granulation crosslinker. To add cells to the non-granular hydrogel, the cells were added when the hydrogel and crosslinking agent were combined and then the hydrogel was allowed to crosslink with embedded cells.

3.4.2. Non-Granular Hydrogel Cell Seeding Characterization

Non-granular hydrogels were prepared at 1% (w/v) concentration with NBM supplemented with L-glutamine, 2% (v/v) neuronal stem cell supplement, 1% (v/v) P/S, 1% (v/v) NEAA, 10 ng/ml (0.38 nM) NGF, 50 ng/ml (3.6 nM) BDNF, 20% (v/v) crosslinker, and RA treated SH-SY5Y neuroblastoma cells at 1×10^6 cells/ml. Two experimental groups were prepared by adding laminin at 20 $\mu\text{g/ml}$ (0.02 μM) and 200 $\mu\text{g/ml}$ (0.22 μM) to the above mixture. 70 μl of cell-seeded hydrogel was transferred to each of three 8 mm diameter mini-wells created by inserting PDMS O-rings into the 24 well plate. The hydrogel constructs were allowed to set for 30 minutes. A 2D control set was prepared by seeding cells at 1×10^5 cells/cm² into 3 wells of a 24 well plate previously incubated for 60 minutes with a sterile solution of 2.5 $\mu\text{g/cm}^2$ laminin in PBS. Cells were allowed to proliferate for 14 days, exchanging medium every 2 days. Live-dead (Invitrogen, UK) assays were taken on days 7 and 14. The live-dead images were captured with widefield imaging, and the cell count was measured using Imaris 3D image processing software (Bitplane, Switzerland).

3.4.3. Scaffold Stability and Cell Clustering Characterization

Granular 2% (w/v) hydrogels were prepared supplemented with 1.25 $\mu\text{g/ml}$ (90 nM) BDNF, and seeded 1:1 (v/v) with NBM supplemented with L-glutamine, 2% (v/v) neuronal stem cell supplement, 1% (v/v) P/S, 1% (v/v) NEAA, 10 ng/ml (0.38 nM) NGF, 50 ng/ml (3.6 nM) BDNF, laminin at 300 $\mu\text{g/ml}$ (0.33 μM), and GFP modified RA treated SH-SY5Y neuroblastoma cells at 2×10^7 cells/ml, and 2%, 4%, 6%, 10%, 15% and 20% (all v/v) post-granulation crosslinker (PGC). PGC group samples were prepared in triplicate. Cellularized scaffolds were allowed to remain in culture for 28 days, with widefield fluorescence imaging every 7 days to monitor and analyse hydrogel breakdown over time. ImageJ length analysis was used to measure the change in width of the scaffolds over time.

3.4.4. Granular Hydrogel Granule Size Characterization

Acellular 2% (w/v) hydrogels were prepared and granulated through the 40 μm , 17 μm , and 13 μm mesh sizes before placing a small amount ($\sim 100 \mu\text{l}$) of the hydrogel in a tilted petri dish, to allow spatial separation of the granules. Using DIC microscopy, samples were imaged, and using ImageJ software length analysis, hydrogel granule diameters for at least 30 granules per group were calculated by averaging three edge-to-edge measurements, taken across three axes drawn through the central point of the granule, rotated approximately 120° from each other around the central point.

A 1 ml volume of granules prepared using 2% (w/v) hydrogel extruded through the 17 μm mesh were placed into a tilted petri dish to allow granule separation. The dish was placed on the microscope stage and the image was focused onto granules in the field of view, before adding 1 ml PBS to the petri dish. Images were captured using DIC imaging every 30 seconds for 100 minutes. The change in granule diameter of seven granules was measured and analysed using ImageJ software length analysis.

Granular hydrogels prepared at 2% (w/v) concentration with the 40 μm , 17 μm , and 13 μm sieve sizes, supplemented with 1.25 $\mu\text{g/ml}$ (90 nM) BDNF, were seeded 1:1 (v/v) with NBM supplemented with L-glutamine, 2% (v/v) neuronal stem cell supplement, 1% (v/v) P/S, 1% (v/v) NEAA, 10 ng/ml (0.38 nM) NGF, 50 ng/ml (3.6 nM) BDNF, laminin at 0.3 g/ml (0.33 μM), 4% (v/v) post-granulation crosslinker, and RA treated SH-SY5Y neuroblastoma cells at 2×10^7 cells/ml. Three aliquots of 70 μl of hydrogel per group were placed onto a glass slide using a 1 ml syringe, before pressing the hydrogels to a 1 mm thickness using another glass slide with attached 1 mm silicone separator. The hydrogel constructs were allowed to set for 30 minutes. Then, the seeded hydrogel constructs were transferred from the glass slides to wells of a 24 well plate where they were allowed to proliferate for 14 days, exchanging medium every 2 days. Live-dead (Invitrogen, UK) assays were taken on days 7

and 14. The live-dead data was processed and the cell count was taken using Imaris 3D image processing software (Bitplane, Switzerland).

3.4.5. Hydrogel Rheology Characterization

Acellular 1% (w/v) and 2% (w/v) non-granular hydrogel constructs were prepared as previously described (section 3.4.1), without the addition of cells or media, and analysed for time lapse gelation rheology using an oscillatory rheometer (Anton Paar, UK). A 500 μ l total volume of each hydrogel sample was reconstituted and placed between the two plates just before starting a time lapse of gelation at 1 Hz frequency oscillation over 30 minutes.

The DWS machine was setup to collect transmission laser beam light scattering measurements at 37°C and 101.3 kPa pressure (NTP). Polystyrene (PS) beads were added to the calibration solution as well as to the samples to facilitate measurement of light scattering due to Brownian motion of the particles. This collected data was then used by the DWS machine to calculate storage and loss modulus measurements.

The DWS machine was calibrated with a 1% (w/v) 200 nm diameter PS microbead solution in water (Sigma Aldrich, UK), in a 2 mm thick cuvette, before collecting storage modulus (G') and loss modulus (G'') values over 30 seconds of multi tau sampling and 30 seconds of echo sampling to collect a full range (~ 0.01 -20,000 Hz) frequency sweep at each time point for the hydrogel samples. Acellular 1% (w/v) and 2% (w/v) non-granular and granular hydrogel constructs were prepared and analysed for static rheology and time lapse rheology using the Diffusing Wave Spectroscopy (DWS) microrheology machine (Merrow Scientific, UK).

Acellular hydrogels were reconstituted according to described protocols (section 3.4.1), modified to include 10% (v/v) 200 nm diameter PS microbeads in solution (10% (w/v), H₂O, Sigma Aldrich, UK) for a final concentration of 1% (w/v) PS microbeads in the hydrogel.

Specifically, the 1% (w/v) and 2% (w/v) non-granular hydrogels were prepared with addition of 10% (v/v) PS bead solution, and without the addition of cells or media, and the 1% (w/v) and 2% (w/v) granular hydrogels were prepared by granulating these non-granular hydrogels of 1% (w/v) and 2% (w/v) concentration respectively.

For gelation rheology characterization, a volume of 400 μ l of 1% (w/v) and 2% (w/v) non-granular hydrogels was prepared, adding the crosslinking agent and placing in 2 mm thick cuvettes immediately before collecting time lapse gelation rheology measurements in triplicate over 30 minutes using DWS. The three hydrogel samples were allowed to crosslink for 24 hours at 37°C before collecting static rheology measurements.

For granulation rheology characterization, non-granular hydrogels were crosslinked for 24 hours at 37°C, granulated, and 400 μ l of the hydrogel was delivered into the 2 mm cuvette using a syringe before collecting static rheology measurements in triplicate.

Statistical analysis was carried out on the 24 hour crosslinked 1% (w/v) and 2% (w/v) NGG and GG bulk rheology dataset using ANOVA single factor test, with $p < 0.05$ as the total threshold for statistical significance. Four Bonferroni corrected post-hoc student's t-tests were used to analyse further differences between groups, with $p < 0.013$ selected as the adjusted threshold for statistical significance.

For granular hydrogel swelling and PGC rheology characterization, a 200 μ l volume of 2% (w/v) granular hydrogel was combined with 200 μ l PBS and 17 μ l PGC in the 2 mm thick cuvette immediately before collecting time lapse rheology measurements of granule swelling and post-granulation crosslinking over 120 minutes.

3.4.6. Hydrogel Cell Seeding

A 2D control set was prepared by seeding GFP RA differentiated SH-SY5Y cells at 1×10^5 cells/cm² into 3 wells of a 24 well plate previously incubated for 60 minutes with a sterile solution of 2.5 µg/cm² equivalent concentration of laminin in PBS.

Non-granular hydrogels were prepared at 1% (w/v) concentration with NBM supplemented with L-glutamine, 2% (v/v) neuronal stem cell supplement, 1% (v/v) P/S, 1% (v/v) NEAA, 10 ng/ml (0.38 nM) NGF, 50 ng/ml (3.6 nM) BDNF, 0.3 g/ml (0.33 µM) laminin, 20% (v/v) crosslinker, and GFP RA treated SH-SY5Y neuroblastoma cells at 2×10^7 cells/ml. 70 µl of cell-seeded hydrogel was transferred to each of three 8 mm diameter mini-wells created by inserting PDMS O-rings into the 24 well plate. The hydrogel constructs were allowed to set for 30 minutes before adding 2 ml supplemented NBM.

Granular hydrogels prepared at 2% (w/v) concentration using the 40 µm sieve size and supplemented with 1.25 µg/ml (90 nM) BDNF were seeded 1:1 (v/v) with: NBM supplemented with L-glutamine, 2% (v/v) neuronal stem cell supplement, 1% (v/v) P/S, 1% (v/v) NEAA, 10 ng/ml (0.38 nM) NGF, 50 ng/ml (3.6 nM) BDNF, laminin at 300 µg/ml (0.33 µM), 4% (v/v) post-granulation crosslinker, and GFP RA treated SH-SY5Y neuroblastoma cells at 2×10^7 cells/ml. Three aliquots of 70 µl of hydrogel were placed onto a glass slide using a 1 ml syringe, before pressing the hydrogels to a 1 mm thickness using another glass slide with attached 1 mm silicone separator. The hydrogel constructs were allowed to set for 30 minutes. Then, the seeded hydrogel constructs were transferred from the glass slides to wells of a 24 well plate containing 2 ml supplemented NBM.

All samples were maintained for 14 days with 2 ml/well NBM supplemented with L-glutamine, 2% (v/v) neuronal stem cell supplement, 1% (v/v) P/S, 1% (v/v) NEAA, 10 ng/ml (0.38 nM) NGF, 50 ng/ml (3.6 nM) BDNF, and exchanging medium every two days.

3.4.7. *Image Collection and Analysis*

Live-dead assays were taken on days 7 and 14. The live-dead data was processed and the cell count was taken using Imaris 3D image processing software. Cells were fixed in 4% (v/v) paraformaldehyde in PBS and stained on days 7 and 14 for tubulin and nucleus, and imaged using confocal microscopy.

Live imaging was taken for 1.5 hour sessions on day 6 and day 12 and this data was also processed for neurite length and connectivity using ImageJ software. Neurite length was calculated by measuring the average total length of 70 selected neurites, neurite connectivity was calculated by measuring the average number of neurons extending neurites and forming neuron-neuron connections as a percentage of the number of neurons in view, and neurite extension was calculated by measuring the average change in total neurite length for 10 selected neurites at 10 minute intervals over 60 minutes. For neurite length and connectivity, statistical analysis was carried out on the whole dataset using ANOVA single factor test, with $p < 0.05$ as the total threshold for statistical significance. Three Bonferroni corrected post-hoc student's t-tests were used to analyse further differences between groups, with $p < 0.017$ selected as the adjusted threshold for statistical significance.

3.5. **Development of a Granular Hydrogel Tertiary (3°) Extrusion Device**

3.5.1. *Tertiary Extrusion Device Design and Printing*

Two subsequent versions of a hydrogel extrusion device, with modified multi-luer-lock design, were prepared using Autodesk Inventor Professional 3D design software. The design was adapted from parts from a database prepared by Dr Julian George, of the author's lab. The device was printed using durable resin at 0.1 mm resolution on a stereolithography 3D printer (Formlabs, UK). The sample was prepared for printing by orienting vertically (inputs

above output) on the Form Labs software print stage, then rotating 20° around the x-plane and 40° around the y-plane, and adding external supports at full density and 0.7 mm touchpoint size. Post printing, the samples were washed by soaking in 100% ethanol (Sigma Aldrich, UK) for 10 minutes, allowed to dry, and finally allowed to light and heat cure at 60°C and 405 nm wavelength light for 60 minutes.

3.5.2. Fluid Modelling

The input and output paths from version 1 were identified and separated from the rest of the device design, and transformed to a solid path with an extended output length for the purposes of simple fluid laminar flow modelling using ANSYS Workbench software (Ansys, UK). The input velocity for both entry points was selected at 0.01 m/s, and output gauge pressure was selected at 0 Pa. No slip shear conditions were implemented on stationary walls, and least squares cell based solution method was run with a fluid of 1140 kg/m³ density and 30 kg/m.s viscosity. The solution was plotted using pathlines (line-arrows) released from and colour coded to the inputs to qualitatively assess the movement and distribution of fluid from the two inputs as they progressed to the output.

Results from fluid modelling of version 1 informed the adaptations for version 2, and the modelling process was repeated using the same procedure and modelling conditions as were used for version 1.

3.5.3. Hydrogel Tertiary Extrusion Characterization with Fluorospheres

Acellular 2% (w/v) granular hydrogels were prepared in two batches with 0.4% (v/v) red fluorospheres and blue fluorospheres at 0.5 µm diameter (Molecular Probes, UK). The fluorospheres were added before crosslinking and the seeded hydrogels were then delivered through both versions of the tertiary (3°) extrusion device into petri dishes and samples were maintained at -80°C for 72 hours. Using a scalpel, 1-2 sections were carefully and quickly

removed from the middle of each sample, placed transverse section down onto glass bottomed petri dishes (ThistleScientific, UK), and imaged using confocal microscopy. Images were analysed using ImageJ software to measure integrated density of red and blue fluorescence in each quadrant of the samples and quantify the interspersions of cellular and acellular hydrogels.

3.5.4. *SH-SY5Y Cell Seeding Experimental Setup*

Granular hydrogels at 2% (w/v) concentration were prepared in two batches. Red granular hydrogel was prepared with 0.13% (v/v) red fluorospheres at 0.5 μm diameter and green granular hydrogel was prepared with 0.13% (v/v) green fluorospheres at 0.5 μm diameter and 1.25 $\mu\text{g/ml}$ (90 nM) BDNF. Green granular hydrogel was combined 1:1 (v/v) with RA treated SH-SY5Y cells suspended at 4×10^7 cells/ml in neurobasal medium, supplemented with 300 $\mu\text{g/ml}$ (0.33 μM) laminin, 50 ng/ml (3.6 nM) BDNF, 10 ng/ml (0.38 nM) NGF, and 4% (v/v) crosslinker (cellular hydrogel). Red granular hydrogel was combined with 4% (v/v) crosslinker only (acellular hydrogel). The prepared hydrogels were then placed into separate syringes and attached to the 3° extrusion device input openings before extruding three hydrogel samples through version 2 of the 3° extrusion device onto a petri dish. A non-3° extruded sample set was prepared by combining the prepared red and green hydrogels 1:1 (v/v) in a 2 ml microcentrifuge tube, mixing, and delivering three samples into the petri dish using a syringe as the transfer device. Hydrogels were allowed to cure for 30 minutes from the addition of PGC to the hydrogel and then 3 ml media was added to each petri dish. Cells were allowed to proliferate in the hydrogel scaffolds for 10 days, exchanging media every two days. On day 5 and day 10, Hoechst stain (NucBlue Live, ThermoFisher Scientific) was added to the samples and allowed to incubate for 30 minutes. Samples were imaged using confocal microscopy and analysed using ImageJ software to measure the cell nucleus distribution within the cellular and acellular hydrogel granules.

The experiment was repeated using cellular and acellular granular hydrogels without fluorospheres, prepared and seeded with otherwise the same procedure as was done with the fluorescently marked hydrogels. These hydrogel scaffolds were maintained for 14 days, exchanging media every two days. On days 7 and 14, samples were fixed and immunohistochemistry was carried out, staining for tubulin and nucleus before capturing confocal images for analysis of cell distribution (density) and alignment within the hydrogel scaffold. Statistical analysis was carried out on the 3° extruded and non-3° extruded cell density datasets using ANOVA single factor test, with $p < 0.05$ as the total threshold for statistical significance. For cell density data, three post-hoc Bonferroni corrected student's t-tests were then carried out to analyse significances between groups, with $p < 0.017$ as the adjusted threshold for statistical significance.

3.5.5. *F11 Cell Seeding and Differentiation*

Granular hydrogels at 2% (w/v) concentration were prepared in two batches: with and without 1.25 $\mu\text{g/ml}$ (90 nM) BDNF. Granular hydrogel with BDNF was combined 1:1 (v/v) with F11 neuroblastoma cells suspended at 4×10^7 cells/ml, in complete medium composed of DMEM high glucose, supplemented with 1% (v/v) FBS, 1% (v/v) P/S, 2 μM RA, 10 μM 3-isobutyl-1-methylxanthine (Sigma Aldrich, UK), 250 μM insulin transferrin selenium (Gibco, UK), 0.5 mM 8-br-cAMP (Sigma Aldrich, UK), 50 ng/ml (1.9 nM) NGF, and 4% (v/v) crosslinker (cellular hydrogel). Granular hydrogel without BDNF was combined with 4% (v/v) crosslinker only (acellular hydrogel). The prepared hydrogels were then placed into separate syringes and attached to the 3° extrusion device input openings before extruding three hydrogel samples through the 3° extrusion device onto a petri dish. A non-3° extruded hydrogel sample set was prepared by combining the prepared hydrogels 1:1 (v/v) in a 2 ml microcentrifuge tube, mixing, and delivering three samples into the petri dish using a syringe as the transfer device. Hydrogels were allowed to cure for 30 minutes from

the addition of crosslinker to the hydrogel and then 3 ml media was added to each petri dish. A 2D control set was prepared by seeding cells at 1×10^5 cells/cm² into 3 wells of a 24 well plate previously incubated for 60 minutes with a sterile solution of equivalent concentration for 2.5 µg/cm² laminin in PBS. Cells were allowed to proliferate in the hydrogel scaffolds for 5 days, exchanging media every day.

3.5.6. F11 Sample Calcium Imaging

On day 5, calcium imaging was performed on the samples. Cells were incubated with 4 µM Oregon Green 488 BAPTA-1, a high affinity cell permeable calcium indicator, in PBS for 35 minutes (OGB-1, ThermoFisher Scientific UK). Cells were then washed thrice for 1 minute each time in PBS before being incubated with live-cell imaging media (ThermoFisher Scientific, UK) for another 35 minutes. Samples were transferred to an enclosed heated and gassed microscope stage top chamber to undertake imaging using widefield fluorescence, capturing images at 100 – 300 ms exposure time with no delay between images, for 3 minutes. Videos were analysed using high-speed review, and regions of interest were selected to measure fluorescence of the firing cells over the time period. Spike rate and maximum spike intensity was measured and calculated for 10 cells per group. Statistical analysis was carried out on the whole dataset using ANOVA single factor test, with $p < 0.05$ as the total threshold for statistical significance. Three Bonferroni corrected post-hoc student's t-tests were used to analyse further differences between groups, with $p < 0.017$ selected as the adjusted threshold for statistical significance.

Chapter 4: Optimizing the Microporous Interface for Human Neural Network Culture

4.1. Chapter Introduction

This chapter investigates the manipulation of topographic cues on microporous, flat scaffold structures. The experiments are motivated to manipulate contact guidance of neurites to direct the network formation, and use microfabrication to guide neurite growth into funnel-shaped micropores. The findings from this chapter not only improve the theoretical understanding of the interaction of neurons with their surrounding environment, but also advance the development of a low density neural interface.

Firstly, polycarbonate track etched membranes (figure 4.1A, C) were seeded with RA differentiated SH-SY5Y cells, and the interaction of the cells with pores on the membrane was observed using high magnification imaging. These porous membranes were shown to direct neurite progression through the pores at high cell seeding density. Following this, ordered microporous surfaces with sloped pore entry, allowing interaction with the pore at the desired angle at all entry points around the horizontal surface (funnel-shaped microporous glass coverslips, figure 4.1B, D) were designed, machined, characterized, and optimized. Optimization of the procedure resulted samples in two angle groups, and while the investigated annealing process did not result in reduced surface roughness, samples were adequate to undertake investigative cell-seeding experiments. From these experiments, it was found that shallower funnel-shaped pores were better able to direct neurite progression, compared with steeper funnel-shaped pores. This control over pore shape and size improved the controllability of low density seeded cultures.

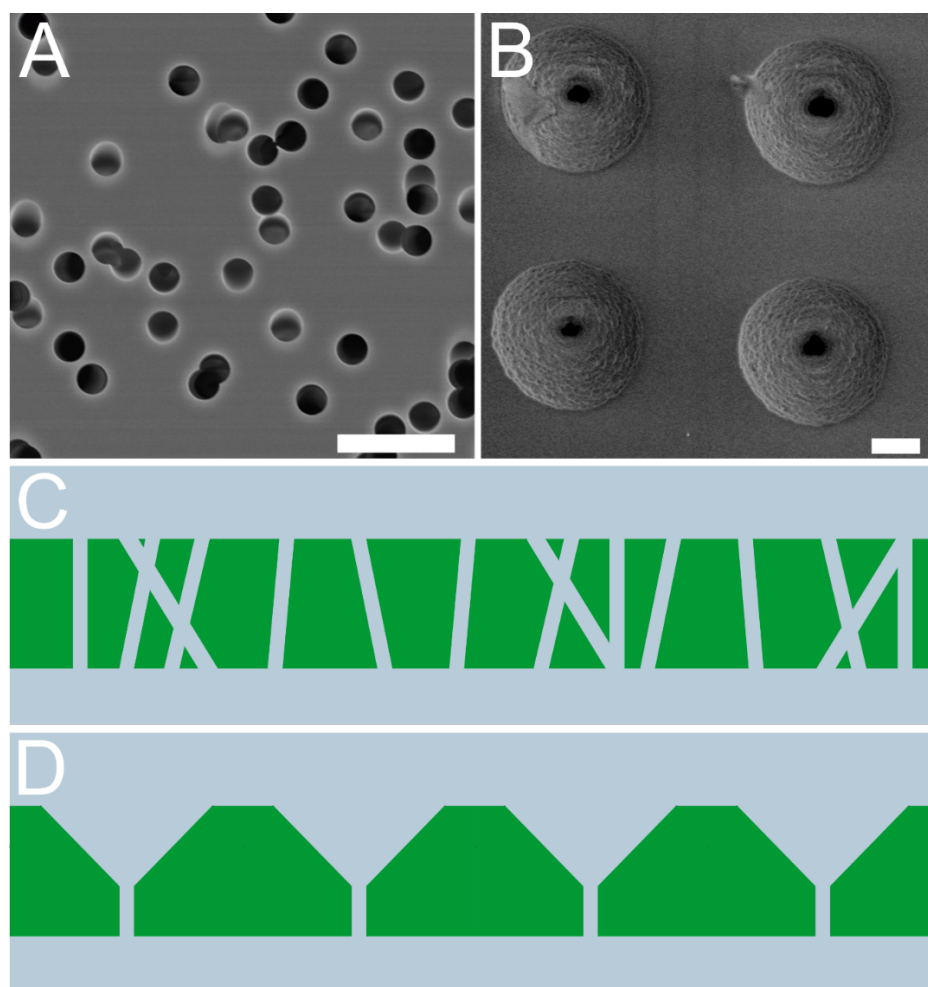


Figure 4.1. The polycarbonate and funnel-shaped micropores. **A.** SEM image of the 1.2 μm polycarbonate track etched membrane. Scale bar = 5 μm . **B.** SEM image of the funnel-shaped micropores on a glass coverslip. Scale bar = 30 μm . Schematics of the section view of **C.** the polycarbonate track etched membrane and **D.** the funnel-shaped micropores on a glass coverslip, showing a move from disordered pores machined at various angles, to ordered pores machined with shallow sloping pore entry.

4.2. Polycarbonate Track Etched Membranes

4.2.1. Seeding Density and Neurite Progression

High magnification imaging of the polycarbonate membranes show that as the neuron growth cones explore the environment and interact with the vertical pores, they do not preferentially progress through the pores (figure 4.2, supplementary figures S2-5). Instead, the cells approach the pores and subsequently move away from them and across the membrane surface.

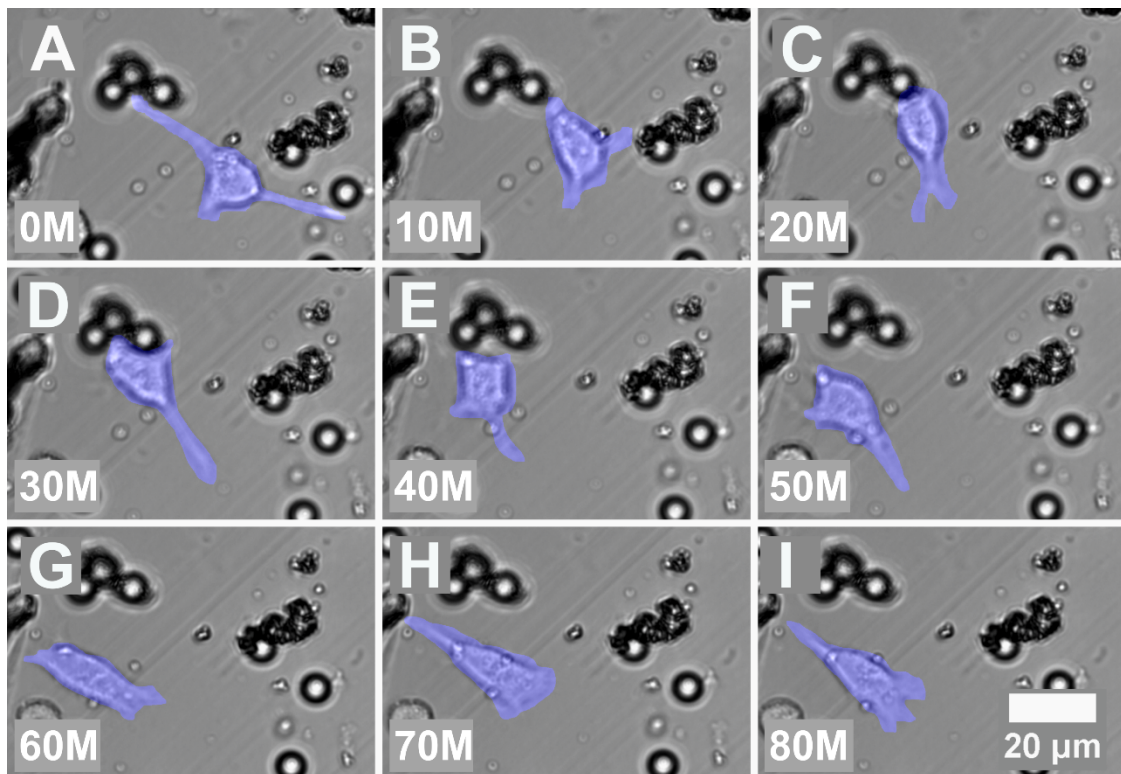


Figure 4.2. Still images showing the progression of an RA treated SH-SY5Y cell seeded at low density (1×10^4 cells/cm²) on an 8 μm polycarbonate track-etched porous membrane. As the neurite growth cone approaches the right-angled/steeply angled pores, it does not progress into and through the pores but rather progresses around and away from them. Images progress from A to I with ten-minute intervals.

This can be explained by the phenomenon of contact guidance. As the growth cone of the neurite approaches the edge of the pore, the restriction of motility of the bending cone results in a decision to no longer move towards the pore edge. This can be further explained as seen in Ferrari et al., where neurites were directed to grow along nanotracks [106]. The authors observed that the microtubules and microfilaments that make up the growth cones preferentially formed along the aligned direction of the nanogratings, however, for cells seeded on a typical, non-aligned two dimensional surface, the microtubules and microfilaments, and therefore the growth cones, were formed in all directions. It is likely that on a microscale level, as the microtubules are being remodelled, they sense the steep angular topographical environment and make decisions to continue or cease progression, directing the movement of the growth cone, neurite, and cell [107].

Percentage area coverage of the neurites on the underside of the membrane was measured and quantitative results indicate that high density seeded cultures are necessary for neurite progression through the pores. At lower density cell seeding numbers, neurite progression through the pores is reduced. Image analysis of the underside of the track-etched membranes showed that neurite growth through the pores was significantly decreased (less than 2% area coverage) for seeding densities below 1×10^5 cells/cm². Above this seeding density, neurite growth through the membranes increased relative to the seeding density (figure 4.3). These results indicate that there is a seeding density threshold below which the neurite growth through the membrane is impeded. When the cells are seeded at low density, the neurite growth cones experience the microporous surface with the ability to explore and move along the horizontal surface avoiding the steep surfaces of the pore edges [108], [109]. However, as the cell density increases, cells become surrounded by other cells on the horizontal surface, resulting in a reduction of the available horizontal surface for the cell's proliferation.

Therefore, neurite growth cones are forced to explore and move along and into the pore edge as these paths become the more preferential topography.

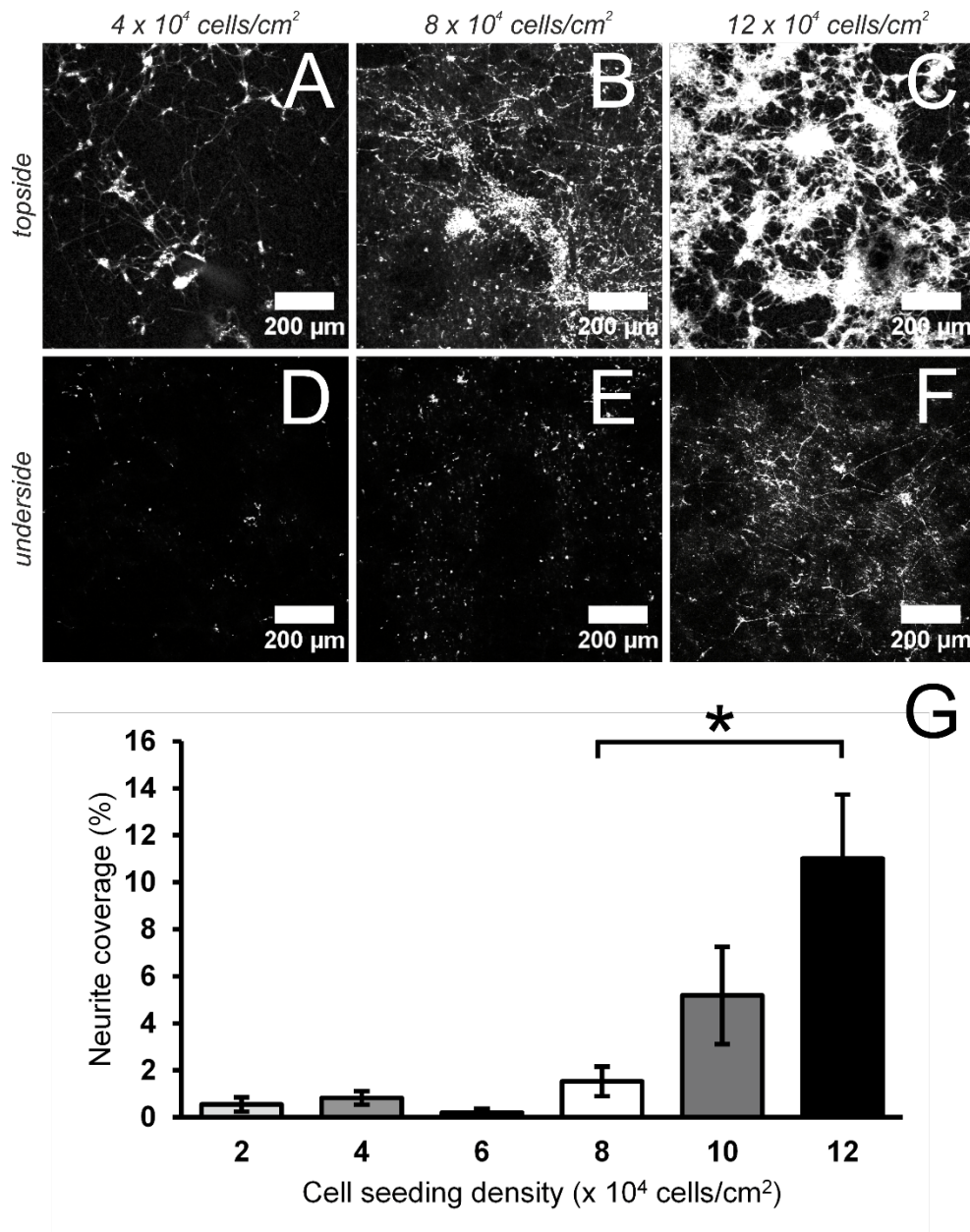


Figure 4.3. Quantification of neurite progression. *A – F.* Maximum intensity projection fluorescent images of the polycarbonate track etched membranes at select seeding densities. There is little to no neurite outgrowth on the membrane underside for seeding densities below 1×10^5 cells/cm². *G.* Column graph showing resulting neurite coverage as determined using image analysis on the underside of the track-etched membrane for different cell seeding densities. Neurite progression through the membrane is significantly reduced for seeding densities below 1×10^5 cells/cm². ANOVA, $p < 0.05$, * Bonferroni corrected student's *t*-tests, $p < 0.017$. $N = 48$, error bars represent standard deviation.

These results are important, especially considering the application of this neural network to act as a cellular neural interface. Such a cell network would need to be cultured at low density and non-confluence in order to maintain the capability for specific targeting of the network. At lower density, the cell network would contain fewer overlapping cell-cell connections across the top layer, resulting in a simpler format for input stimulation and better access to individual cells in the network. Stimulation of high density, confluent cultures on the other hand, is likely to result in unpredictable neural firing, and subsequent difficulty directing the input into the system [110], [111]. Therefore, cultures created by these polycarbonate track etched membranes are likely to be unsuitable for the neural interfaces, acknowledging that confluency is necessary for these cultures to develop neurite growth through to the opposite side of the membrane.

Given these results, it was hypothesized that to prepare low seeding density cultures, laser machining of angled topography in the form of funnel-shaped pores would preferentially direct the growth of neurons through the pores, rather than relying on confluency on the top layer to force neurons to progress to the opposite side due to physical constraints. Angled topography in the form of a funnel shaped pore entry would facilitate neurite entry around the entirety of the circumference of pore entry. Furthermore, the phenomenon of contact guidance supports the hypothesis that a shallower angled pore entry could promote neurite entry into the pore without the need for confluency, while previous experiments indicate that the narrow pore exit ($< 2 \mu\text{m}$) would restrict cell bodies of the neurons to remain on the top layer, forming a separate yet connected neural interface layer [112]. And finally, laser machining would allow for tight control of positioning and morphology of the funnel-shaped pore across the interface surface, and subsequently allow for user determination of the stimulation access points.

The optimization and implementation of the chosen protocol, preparing funnel-shaped pores to assess this hypothesis, is investigated in the following section. This protocol was designed with the intention of application to prototype development as well as full-scale manufacture development. Therefore, feasibility was determined by level of achievement of a quick, simple, and scalable technique, producing samples of low variability and high accuracy in the feature dimensions.

4.3. Funnel-Shaped Microporous Glass Coverslips

4.3.1. Procedure Optimization

Scanning electron microscopy of the unseeded funnels showed that progress was made in optimizing the laser machining and etching process. At the first iteration, SEM showed that there was a remnant piece of glass in the centre of the funnels (figure 4.4A). This result prompted a change in the laser machining process: from one that ablated the walls of the funnels only, to one that ablated the walls of the funnel as well as the entire volume inside the funnel shape. Resulting funnels contained some preferentially etched ridges in certain areas of the funnel (figure 4.4B). After a second restructure of the laser ablation process, the laser power was changed from constant power to variable power, increasing the power as the laser progressed downward through the vertical distance. The vertical distance between ablated layers was also reduced. SEM analysis of subsequent iterations showed that there was no longer a remnant piece of glass, or a preferentially etched ridge in the funnels (figure 4.4C). Over the iterations, the funnel obtained topography closer to the expectations.

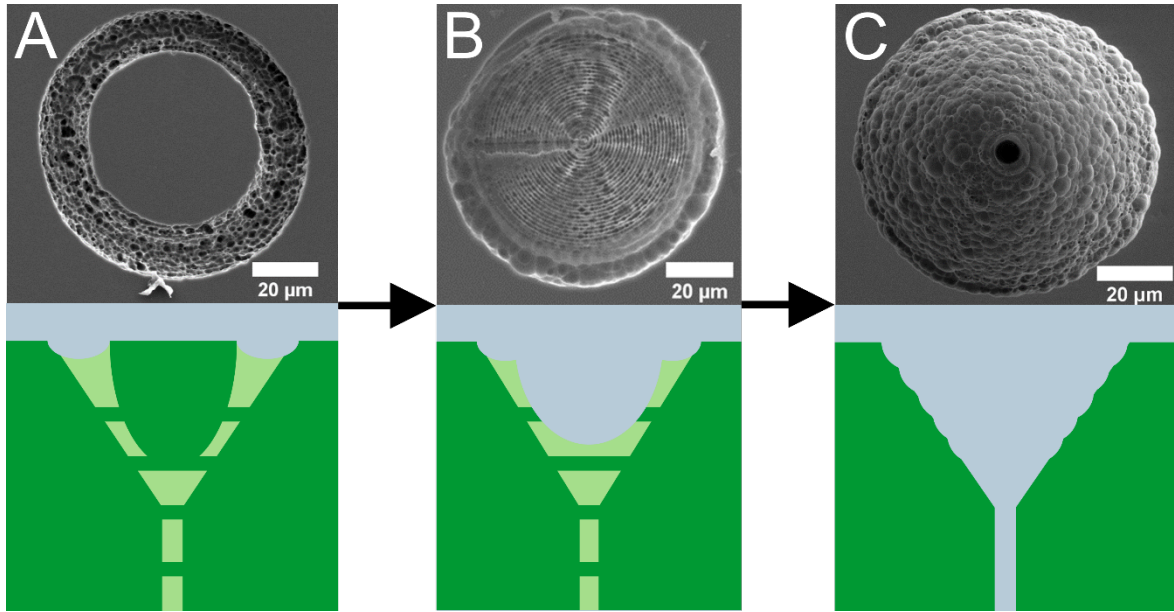


Figure 4.4. SEM images (upper row) and schematics of section view (lower row) showing morphological progression of the funnelled pores. **A.** Initial iteration implementing wall only ablation, at constant laser power, resulting in glass remnant at the centre of the funnel shape. **B.** Iteration implementing full volume ablation, at constant laser power, resulting in ridges. **C.** Final iteration implementing full volume ablation, at increasing laser power, and closer vertical layer distance. With each iteration, the funnel becomes more and more as expected. Dark green areas represent unablated glass, light green areas represent ablated, unetched glass. Scale bars = 20 μm .

4.3.2. Angle and Funnel Diameter Characterization

For the funnel-shaped pore features that were imaged using Alicona InfiniteFocus profilometry, and resulting profiles showed that, despite differences in fabrication regime, the three intended angle groups were not fabricated. However, by machining the funnels at different depths to the surface, it was possible to create samples of two angle groups (figure 4.5A, B). Average angle measurements for the two angle groups were quantified at $61.6 \pm 9.8^\circ$ and $86.9 \pm 2.0^\circ$ (figure 4.5D). The average funnel mouth diameter for the two angle groups were $74.7 \pm 1.7 \mu\text{m}$ and $71.2 \pm 1.2 \mu\text{m}$ respectively (figure 4.5C). The variation of angle is tight in the steeper angled group (90°), however there is greater variation in the shallower angled group (60°). There were limited samples available that were composed with the bottom pore opening connected to the funnel portion. Moreover, due to the vertical limit of Alicona profilometry, it was not possible to collect data for the diameter of the bottom pore opening using this technology, and using SEM would require sputter coating, altering the surface of the few samples which were reserved for use as experimental samples. For these reasons, the data for bottom pore opening was not collected.

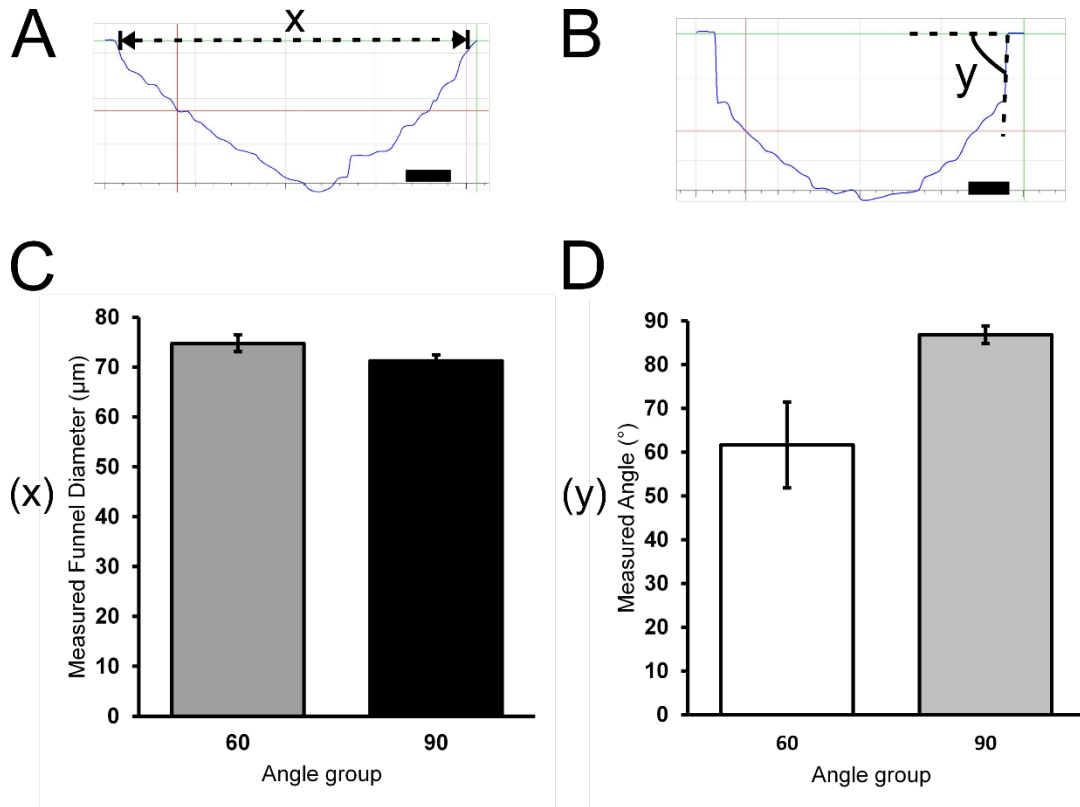


Figure 4.5. Funnel angle and diameter characterization. **A.** Resulting 60 angle group profile showing measured funnel diameter (x) **B.** Resulting 90 angle group profile showing measured angle (y). Scale bars = 10 μm. **C.** Column graph showing measured funnel diameter (x) for the two angle groups. $N = 12$ diameter measurements. **D.** Column graph showing the measured angles (y) for the two angle groups. There is a progression from shallow angles to steep angles as expected. $N = 24$ angle measurements. Error bars represent standard deviation across measurements.

4.3.3. *Annealing: Roughness and Funnel Diameter Characterization*

SEM imaging of the funnels before and after annealing showed that the topography of the funnel surface was rough in appearance (figure 4.6A, B). During the laser machining process, the glass was hit with a high power laser that fractured the glass into small pieces. The use of a pulsed laser beam allowed the process to proceed, but upon etching of the fractured glass pieces using hydrofluoric acid, it could be seen that the remaining surface had increased surface roughness. This raised concerns of its suitability for neural cell culture, considering that neurons are sensitive to the topographical environment [45]–[47]. The machining effect has been seen in other laser ablation fabrication methods such as in work done by Duncan *et al.* where microgrooves were fabricated for the directional growth of osteoblast-like cells [53]. However, the cells interacted with the unmachined, unetched portions of the microgrooves in that study, while in this current study, the microfabrication process is such that the cells have to interact with the machined and etched rough surface.

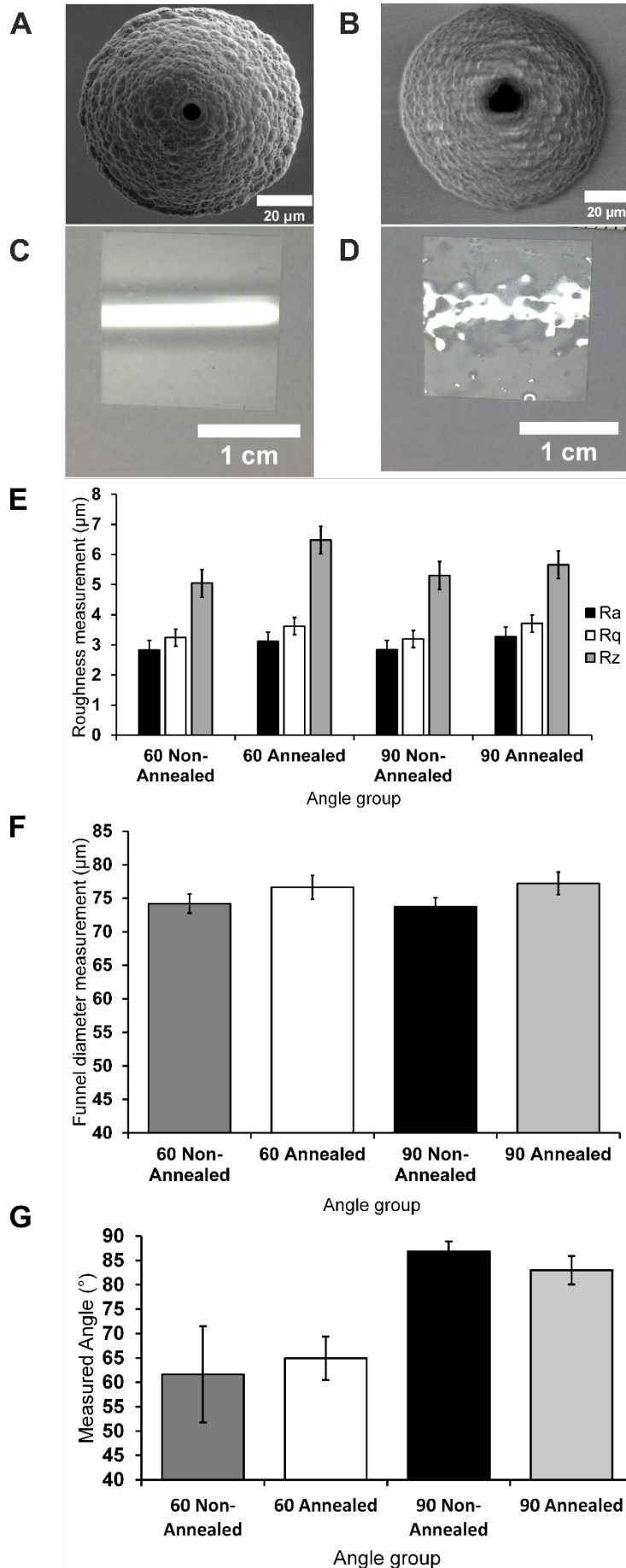


Figure 4.6. Characterization of annealing. SEM images of the 60 angle group funnel surface without annealing (A) and with 1 hour annealing at 500°C (B) and images of the non-annealed 60 angle group coverslip (C) and the annealed 60 angle group coverslip (3 hours at 600°C) (D). Column charts show roughness measurements for the non-annealed and annealed coverslips (E), $N = 144$, funnel diameter for annealed and non-annealed coverslips after 3 hours of annealing at 600°C (F), $N = 40$, and measured funnel angle for annealed and non-annealed coverslips after 3 hours annealing at 600°C (G), $N = 48$. Error bars represent standard deviation across measurements.

Since annealing should allow the glass to reach a temperature that will relieve internal stresses, it was proposed that this process could be used to smooth the microsurface of the glass pores post etch [103]–[105]. However, the data indicates that this may not be the case. After one hour of annealing, there was no visual decrease in roughness (figure 4.6A, B). Similar results were seen for two hours of annealing at 600°C. After three hours of annealing, there was a visible warping of the gross structure of the annealed glass, likely due to the increase thermal energy resulting in enduring rearrangement of the amorphous structure (figure 4.6D) [113]–[115]. Numerical roughness measurements as well as cell culture data indicate no decrease in the microscale surface roughness of the pores.

The numerical results further indicate that the annealing process does not decrease the roughness of the surface. Roughness parameters of mean roughness (Ra), root mean square roughness (Rq) and mean roughness depth (Rz) showed a comparative measure of the roughness of samples in each of the groups tested. These parameters have been used to indicate surface roughness of glass as shown by Kumar *et al.* in their roughness measurements of zinc aluminium oxide glass films [116]. These measurements give an indication of how much variation in height there is across a given surface: the higher the roughness value, the greater the variation across the surface and, therefore, the greater the roughness of the surface. The differences in roughness before and after annealing for 3 hours show that opposite to the expected findings, the roughness measurements indicate a slight increase in the roughness moving from non-annealed samples to annealed samples (figure 4.6E).

The funnel diameter for the non-annealed and annealed groups was measured and compared, and funnel diameter for the annealed 60 angle group was quantified at $76.6 \pm 1.8 \mu\text{m}$, wider compared with the corresponding non-annealed group whose funnel diameter was quantified at $74.2 \pm 1.4 \mu\text{m}$. Similarly, while the non-annealed 90 angle group funnel diameter was

quantified at $73.7 \pm 1.3 \mu\text{m}$, the corresponding annealed funnel diameter was quantified to be wider at $77.2 \pm 1.7 \mu\text{m}$ (figure 4.6F). Although this change in diameter after annealing was only a few micrometres, the standard deviation was tight around the average for all groups, and there was a trend of increase in diameter after annealing across both angle groups. This indicated that along with increasing the surface roughness, the annealing process also increased the size of the funnel-shaped micropore feature. The thermal effects that resulted in warping of the coverslip glass may have also caused the increase in feature size [113]–[115]. Expansion of the glass during the heating process may have resulted in irreversible changes to the funnel-shaped structure, maintaining the widened size upon cooling of the glass.

The funnel angle for the non-annealed and annealed groups was also measured and compared, and small changes in funnel angle were observed after annealing. Non-annealed and annealed 60 angle group funnels were quantified at $61.6 \pm 9.8^\circ$ and $64.9 \pm 4.5^\circ$ respectively, and non-annealed and annealed 90 angle group funnels were quantified at $86.9 \pm 2.0^\circ$ and $83.0 \pm 2.9^\circ$ respectively (figure 4.6G). It is likely that the structural changes in the glass could have resulted in the change in angle measurements. However, with the small differences, high standard deviation, and no clear trend across the angle groups, it is more likely that the difference in angle measurements was rather due to random error associated with the sample differences.

Given that in this study, optical devices are used to measure roughness, it is important to consider whether the values are comparable to those that would have been collected using traditional contact based roughness measurement devices. Taking traditional contact-based roughness measurements is not possible for the micropores because these devices are limited in the vertical distance they can measure. Optical measurement devices such as the Alicona

Infinite Focus machine allow a much wider vertical measurement range. Cuesta *et al.* speak to the accuracy of optically derived roughness measurements at the scanning density [117]. At high scanning densities the optical measurement is at a standard deviation fulfilling ISO standards. This indicates that the optical devices work as well as traditional contact devices under certain conditions. Durabaska *et al.* also studied the suitability of optical measurements of roughness when compared to stylus type profilometers [118]. This study found that for flat surfaces the measurement was comparable while for spherical surfaces the optical measurement and stylus measurement differed significantly. Considering that the optical measurements are taken at high density on surfaces that resemble flat surfaces more than spherical surfaces, it is safe to conclude that the optical roughness measurements are reliable and comparable with those that would be collected from traditional stylus based roughness measurement methods.

Investigations led to a conclusion that over the selected range of annealing times and temperatures, the roughness either remained unchanged or slightly increased for the micropores. Visually at the longest investigated time and highest temperature the glass was warped. From these investigations it is evident that the annealing method investigated did not reduce the roughness of the micropore surface. Instead, the process resulted in not only a slight increase of the roughness, but also an increase in the overall feature size.

4.3.4. *Live Cell Imaging Analysis*

High magnification live imaging of the cell networks as they interacted with the funnelled glass coverslip surface showed that GFP modified RA treated SH-SY5Y cells attached and spread across the laminin coated glass surface, forming connected networks after 24 hours of cell culture. Laminin was coated onto the surface at an equivalent concentration of 2.5

$\mu\text{g}/\text{cm}^2$ in PBS, and incubated at 37°C for 60 minutes, after oxygen plasma treatment of the surface at 160 W for 30 seconds at room temperature ($\sim 20^\circ\text{C}$) and 20 Pa pressure. Similar to coating the surface with biocompatible polymers such as poly(L-lysine), oxygen plasma treatment of the surface activates the surface and increases binding affinity of laminin [119]–[122]. Oxygen plasma interacts to make the surface hydrophilic and abundant in free radicals, allowing subsequent strong covalent binding of the biomolecules to the activated surface [122], [123]. Cells were evenly distributed across the surface and displayed elongated morphology with extended neurites connecting to neighbouring neurons as expected for healthy neuronal cultures. Live imaging observations showed cells exploring the glass coverslip surface. Cells in the areas of the glass surface without funnelled topography moved across the horizontal surface in a random motion with no observable directionality. Cells in close proximity to the funnel lip showed movement either towards (approaching and crossing), around (approaching and skirting), or away from, the funnel boundary (figure 4.7A-F, supplementary figures S6-S9). The number of cells approaching and crossing the funnel boundary was quantified and compared with the total number of cells in view to give a percentage interaction, and results showed greater interaction with the non-annealed 60 angle group funnel edges (34.4% interaction) over the non-annealed 90 angle group funnel edges (25.7% interaction) with an 8.7% increase in cell interaction. The annealed funnel edges garnered similar interaction with each other at 25.9% interaction and 24.0% interaction for the 60 and 90 angle groups respectively. While results showed significantly increased interaction with the shallower funnel over the steeper funnel for the non-annealed samples, there was no significant difference for annealed samples (figure 4.7G).

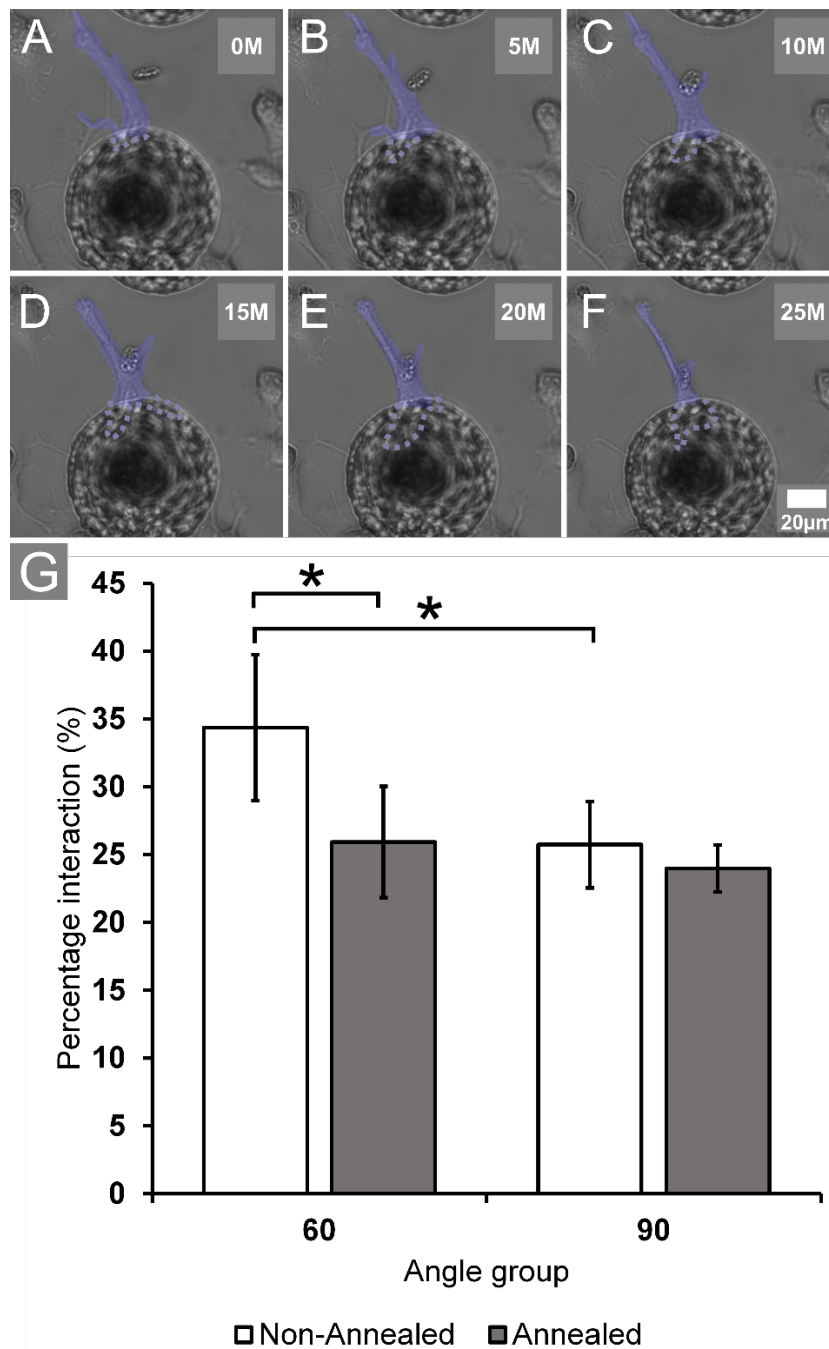


Figure 4.7. Cell interaction with the funnel-shaped pores. **A-F.** Stitched time lapse widefield images showing a neuron approaching and entering a non-annealed 60 angle group funnel. Images were captured 5 minutes apart. Dotted lines estimate cell morphology inside the funnel. **G.** Column chart showing the neuron interaction with non-annealed and annealed 60 and 90 angle group samples. ANOVA, $p < 0.05$, * Bonferroni corrected student's t -test, $p < 0.013$. $N = 346$, error bars represent standard deviation.

The differences in cell interaction with the annealed and non-annealed 60 and 90 angle groups can be attributed to the differences in both the angle of the funnel edge and the surface roughness. Since the non-annealed 60 and 90 angle group samples were recorded having similar surface roughness measurements, it can be proposed that, for these samples, it was the angle difference that carried a greater influence on cell interaction with the topographical surface [19], [46], [50], [106]. As the cells explored the microporous environment they preferentially moved, directed by the growth cone, towards shallower areas and away from steep areas, and this is evident in the increased neuron interaction with the shallower funnel angle.

Concerning annealed samples, while *some cell types are more suited for attachment on rougher surfaces, neurons are particularly sensitive to the surface roughness* of stiff substrates such as silicon and titanium nitride [124]–[126]. In a study by Brunetti *et al.*, investigating neurons on silver coated glass substrates, decreased cell adhesion was observed with increased surface roughness, with only 100 nm difference in roughness compared to the control [127]. *Therefore*, it can be postulated that the increase in surface roughness likely resulted in the decreased cell interaction after annealing, *as the exploring neurons would be less likely to migrate towards areas on the glass surface with lower adhesive properties*. The effect of increase of surface roughness was greater on the annealed 60 angle group samples than on the annealed 90 angle group samples when compared with their non-annealed counterparts, because of the unfavourable steep angular effect already present for the 90 angle groups. Because cells were already directed away from the pore edge in non-annealed 90 angle group samples, the added dissuasive effect of increasing surface roughness within the funnel feature did not decrease the cell interaction as much as observed for the 60 angle group annealed samples compared with their corresponding non-annealed samples.

4.3.5. Feasibility of the Procedure

The motivation for developing laser machined, HF etched micropores was to produce an environment where there was a gradually angled smooth surface to direct the growth of neurites from the top side of the glass to the bottom and separate the neurites from the cell bodies without the need for confluent cultures. This would facilitate creation of a low-density cellular interface layer, separate from yet connected to the representative tissue structure, with the ability to stimulate the connected tissue with high precision. It is evident that the resulting cross section does not provide as smooth an angle as initially desired (figure 4.5, 4.6). This is likely due to the laser ablation manufacturing procedure which creates a sharp transition from surface to funnel. Laser ablation also creates the rough, step like profile which is unintended and can hinder cell motion. It is possible that cell interaction can be improved by preparing a smoother cell interaction surface [128], [129].

While it is worth considering the likelihood that there would be increased cell interaction with a smoother surface, results show that there still was significant difference in cell interaction with the rough funnel surfaces at angles investigated. Moreover, the interaction of cells with the funnel-shaped micropores was greater than what would be expected given the low seeding density (50,000 cells/cm²). For very steep angles on polycarbonate, confluency was necessary for neurite penetration into the pores, however for the funnel-shaped pores, increased cell interaction was seen at low, non-confluent densities, likely due to the use of shallower funnel slopes. This indicates that despite the unfavourable roughness in the surface and the shortfall of the annealing process in reducing surface roughness, the procedure has resulted in samples with a level of suitability for preliminary investigative experiments.

However, there is a second issue, which is that there is variability in etching the pores straight through the coverslip. This variability arises because the manual adjustment of the

coverslip height prior to milling leads to variation in starting positions, and subsequently variation in laser power at corresponding milling depths. In this case it was not possible to use etch time to adequately control the funnel and pore structure. Moreover, it is possible that the glass coverslips used were not of a high enough uniformity in terms of thickness and glass structure, even further affecting the variability of the resulting structures. While it is possible to carry out preliminary investigations with the unfavourable surface roughness, the variability in complete pore feature formation renders the protocol much less feasible in terms of developing scalable prototypes.

Compounding this is the finding that the results from measured funnel-mouth widths are not at a level of repeatability and tight variation suitable for the intended application of cell neurite control. In order to create features which restrict cell movement while allowing the connection of cell processes, it is necessary to have a pore size no larger than 3 μm at the narrowest point [130]–[132]. However, the machining process produces structures at a size 55% greater than the input size, with a variability of 1.4 μm (calculated from the machined and measured funnel widths). At this resolution, it is not possible to create the features as required.

Considering the overall procedure, with the structural improvement over multiple iterations of the laser machining process, and the shortfall of the annealing protocol to reduce surface roughness, it is evident that the current protocol favourably produces samples suitable for testing the fundamental hypothesis of neurite interaction, even though it has not yet produced the intended funnel samples necessary for fully scalable prototype development of a neural interface. From these investigations has come a promising result for experimental development: an indication that these shallow angled funnel-shaped micropores have improved manipulation of low density seeded neural cultures. These findings suggest that, should a protocol be designed that can prepare repeatable, smooth surface funnel-shaped

micropores, there would be potential for future development of distinct, yet connected, neural cultures, useful for fabrication of interfaces to *in vitro* neural models.

4.4. Chapter Conclusion

This chapter investigated the influence of topographic cues on the adhesion and motility of neurites on flat microporous surfaces. For neurite cultures on track etched polycarbonate membranes, with steeply angled pores, it was observed that confluent cell cultures were needed for the neurites to be directed into the pores and that at low density, cells were directed away from the pores rather than into them. Since confluent cultures introduce issues with controllability, the phenomenon of contact guidance was manipulated, to motivate development of a flat microporous scaffold that could direct neurites through the microporous surface at low cell seeding densities.

Analysis of resulting features from the optimized laser machining and HF etching protocol confirmed formation of funnel-shaped pores in two angle groups with shallow and steep angles. However, there was variability in the feature formation straight through the glass, likely attributed to variability in the glass coverslips themselves as well as in the manual adjustment of the coverslips prior to machining. Roughness measurements of the funnel surface indicated that the annealing process did not result in a decrease in roughness. Observation of the live cell interaction of cells with the fabricated micropores showed that there was significantly greater cell interaction at shallower funnel angles compared with steeper funnel angles for the non-annealed samples. It was evident that the annealing process did not improve cell interaction with the funnel edges.

The protocol was intended to be a fast prototyping process that would allow for rapid optimizations of the procedure. However, after investigation, it was concluded that these techniques do not produce the intended funnelled micropore surface at an adequate resolution, nor a quick enough manufacture time, for the procedure to be deemed useful in this way. While the investigated procedure resulted in some difficulty obtaining the intended funnel-shaped micropores, the resulting samples were sufficient to complete live cell analysis and provide insight into the hypothesis that the shallower angled pore entries increase the interaction of neurites with the pores. For this reason, it is still promising that should an alternate, improved protocol be developed to provide more optimal samples, the development of directed cell networks into neural interfaces will be a possibility.

Chapter 5: Granular Hydrogel Scaffolds for Human Neural Network Culture

5.1. Chapter Introduction

This chapter investigates the manipulation of scaffold structure of HA hydrogels, by modifying the internal rheology and adding chemotaxis cues to the 3D scaffold. The experiments use granulation techniques to improve the biocompatibility and 3D network formation within a HA hydrogel scaffold. The findings from this chapter advance development of 3D *in vitro* neural cultures, and provide opportunity for application towards improved artificial nerve graft technology.

Firstly, non-granular hydrogels were seeded with SH-SY5Y cells, and the cell viability and network connectivity were assessed at different laminin concentrations (figure 5.1B). Rounded cell morphology, decreased network connectivity, and decreased cell viability when compared with 2D control cultures seeded onto tissue culture plastic, prompted modifications to the hydrogel gelation process to involve granulation. Granular hydrogel particle and bulk hydrogel properties were characterized and modifications to the gelation process were optimized, and a granular hydrogel scaffold with long term stability was produced (figure 5.1A). Comparison of the cell viability, neurite extension, and network formation within the modified granular hydrogel with the same in the unmodified non-granular hydrogel showed improvement of the neural cultures replicating the control cultures. The modifications made to the hydrogel gelation and cell seeding protocol allowed for the formation of improved neural networks within the three-dimensional scaffold.

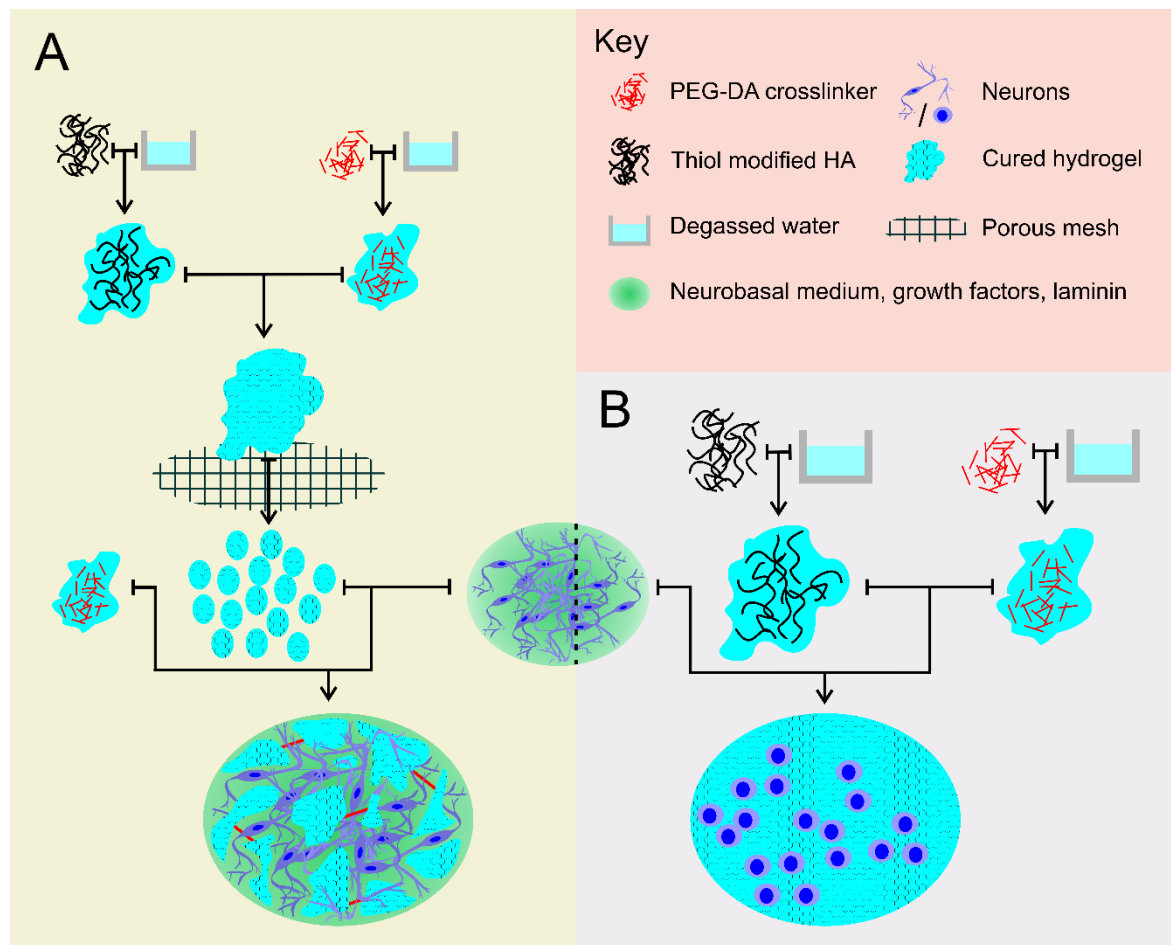


Figure 5.1. Schematic of the hydrogel preparation and cell seeding process. **A.** Preparation of the granular hydrogel scaffold. HA and crosslinker are combined and gelation occurs before granulation and addition of cells and post-granulation crosslinker to the scaffold. **B.** Preparation of the non-granular hydrogel scaffold. HA, cells, and crosslinker, are combined and gelation occurs, encapsulating the cells within a bulk hydrogel.

5.2. Network Characterization in Non-Granular Hydrogels

Visual observation of live-dead stained widefield fluorescence images showed that for the control cultures, cell networks were spread across the entire surface, with healthy neurite connections and little clustering (figure 5.2A). The cells had extended neurites and elongated morphology, similar to what is observed *in vivo*. For non-granular hydrogels at both laminin concentrations, networks were formed with cells clustered together in roughly spherical masses, with few neurite connection between cells within and across cell clusters (figure 5.2B, C). Cells were rounded and did not exhibit the neuronal morphology as observed *in vivo*. There were no visually observable differences in network formation comparing the two hydrogel laminin seeding concentration groups.

Cell viability was calculated as the number of live cells as a percentage of the total number of cells in view. Quantitative analysis showed that the cell viability ranged from 33% to 62% live cells, across all groups and time points (figure 5.2D). Cell viability was decreased in the hydrogel cultures compared with the control cultures, by over 10% live cells at both day 7 and day 14 time points. There was no significant difference in cell viability within the hydrogels seeded with 20 $\mu\text{g/ml}$ (0.02 μM) or 200 $\mu\text{g/ml}$ (0.22 μM) laminin at either time point. Overall, the viability increased from day 7 to day 14 for all groups.

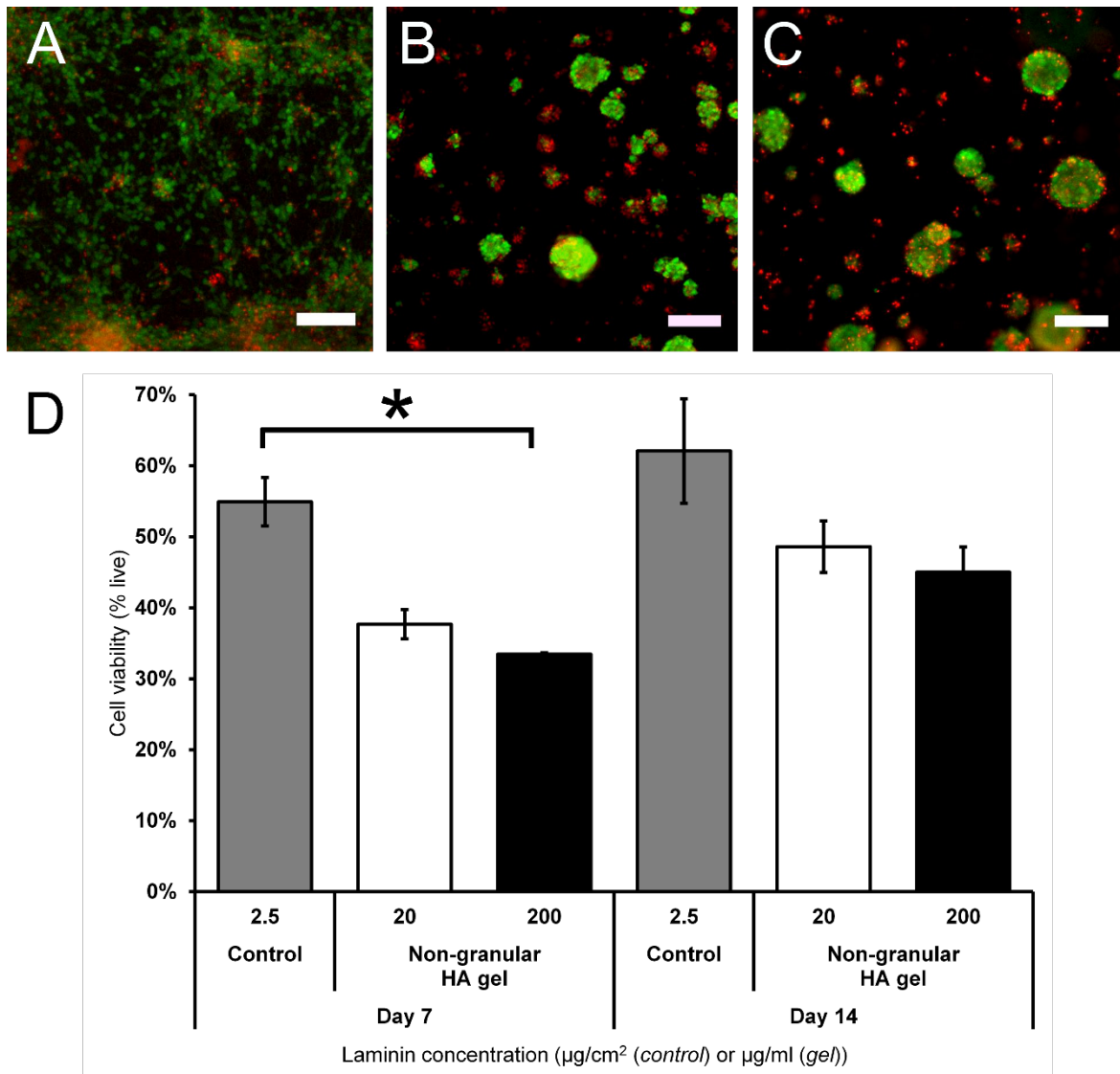


Figure 5.2. Non-granular hydrogel characterization. Day 14 live-dead widefield fluorescence images of the control culture with $2.5 \mu\text{g}/\text{cm}^2$ laminin (A), the non-granular hydrogel with $20 \mu\text{g}/\text{ml}$ ($0.02 \mu\text{M}$) laminin (B), and the non-granular hydrogel with $200 \mu\text{g}/\text{ml}$ ($0.22 \mu\text{M}$) laminin (C). Green represents live cells and red represents dead cells. Scale bars = $150 \mu\text{m}$. **D.** Column graph showing quantified cell viability for the control and non-granular hydrogel cultures over the experimental period. $N = 12$ viability measurements. ANOVA, $p < 0.05$, * Bonferroni corrected student's t -test, $p < 0.013$. Error bars represent standard deviation across measurements.

It was expected that in moving from 2D tissue culture plastic (TCP) cultures to 3D hydrogel cultures, there would be need for an increase in the amount of laminin to correspond with the increase in surface area in the hydrogels. While this experiment was designed to optimize the laminin seeding concentration, results were inconclusive, and moreover, another pertinent issue arose with the observation that neural cultures formed within the hydrogel did not replicate the *in vivo* situation. This finding prompted design and development of a modified hydrogel preparation and cell seeding protocol.

There are several ways that the hydrogel network could be made more representative of the *in vivo* situation in terms of forming more highly connected cell networks. Hyaluronic acid hydrogels are naturally degradable using hyaluronidase enzymatic reactions, reducing agents, and acidic environments, and *in vitro* the hydrogels have been modified with MMP degradable peptides and photosensitive polymers to become specifically degradable for increased cell infiltration and network connectivity [133]–[135]. While these modifications require chemical alterations to the hydrogel structure, physical modification in the form of mechanical granulation of the hydrogel is investigated in this project, to determine whether the modified protocol can result in adequate cell infiltration and network connectivity using a simpler approach.

5.3. Motivation for Development of the Granular Hydrogel: Scaling Up from 2D to 3D

It was hypothesized that preparing a granular hydrogel would allow for areas within the hydrogel to have decreased stiffness while maintaining the hydrated 3D scaffold. These areas of decreased stiffness, between hydrogel granules, would provide an area for cell attachment, morphological elongation, neurite extension, and robust network formation. Additionally, developing a hydrogel of granular nature would allow for computational estimation of the scaffold surface area, and therefore allow for improved estimation of the cell seeding density and laminin concentrations required for the hydrogel per unit volume.

In 2D, the established laminin coating is at 1-3 $\mu\text{g}/\text{cm}^2$ and seeding is at maximum 5×10^4 cells/ cm^2 [51], [136]–[138]. For 3D cultures, in other studies, authors have used up to 4×10^5 cells/ml for 3D seeding and a laminin coating onto the 3D surface of 3-10 $\mu\text{g}/\text{ml}$ [136], [139], [140]. However, if we were to consider the approximate surface area of granular hydrogels, the maths shows we need to increase these parameters.

Granules can be represented as a series of cubes, or of spheres, in the volume space.

For the cube representation:

$$\text{Hydrogel surface area of one } 100 \mu\text{m length cube} = 6 \times (0.01)^2 = 6 \times 10^{-4} \text{ cm}^2$$

$$\text{Number of cubes in 1 ml} = 1 \times 10^6$$

$$\text{Total hydrogel surface area / ml} = 1 \times 10^6 \times (6 \times 10^{-4}) = 600 \text{ cm}^2/\text{ml}$$

$$\text{Laminin coating/ml at } 1 \mu\text{g/cm}^2 = 600 \mu\text{g/ml}$$

$$\text{Seeding density/ml at } 5 \times 10^4 \text{ cells/cm}^2 = 3 \times 10^7 \text{ cells/ml}$$

For the sphere representation:

$$\text{Hydrogel surface area of } 100 \mu\text{m diameter sphere} = 4\pi \times (0.005)^2 = 3.14 \times 10^{-4} \text{ cm}^2$$

$$\text{Number of cubes in 1 ml} = 1 \times 10^6$$

$$\text{Total hydrogel surface area / ml} = 1 \times 10^6 \times (3.14 \times 10^{-4}) = 314 \text{ cm}^2/\text{ml}$$

$$\text{Laminin coating at } 1 \mu\text{g/cm}^2 = 314 \mu\text{g/ml}$$

$$\text{Seeding density at } 5 \times 10^4 \text{ cells/cm}^2 = 1.57 \times 10^7 \text{ cells/ml}$$

Hydrogel granules realistically take a shape in between the sphere and cube. Therefore, approximate hydrogel seeding parameters for the 100 μm granules are a laminin coating at 450 $\mu\text{g/ml}$ (0.5 μM) and cell seeding at 2×10^7 cells/ml. As the granule diameter increases, the total surface area available in the volume decreases (figure 5.3A-C). Subsequently, the seeding density and laminin concentration required for the experimental seeding process also decrease with increasing granule size (figure 5.3D, E). Importantly, these values are much higher than the 2D values, as well as the values that other authors have used in their 3D seeding attempts. We can therefore postulate that increasing the seeding density and laminin concentration can further improve cell culture in 3D hydrogel scaffolds.

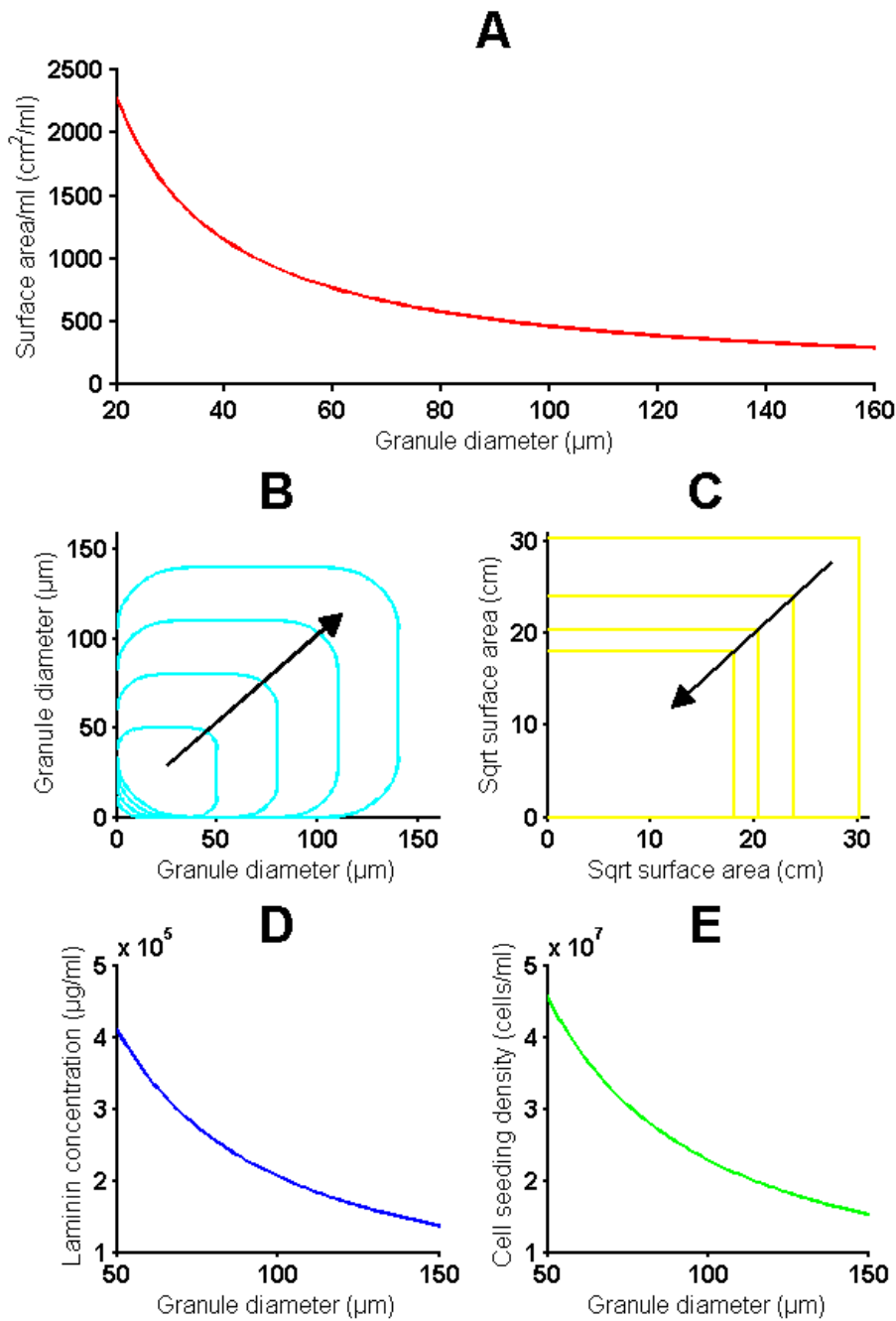


Figure 5.3. Converting 2D to 3D cell seeding and laminin concentration values. **A.** Line graph showing surface area vs. granule diameter. **B, C.** Square graphs showing the increase in granule size resulting in a decrease in surface area/ml. **D.** Laminin concentration vs. Granule diameter and **E.** cell seeding density vs. Granule diameter for the investigated range of granule sizes.

5.4. Hydrogel Preparation and Seeding Protocol Modifications

A number of modifications were made to the HyStem gelation protocol, to prepare the granular hydrogel. Firstly, the protocol was modified from cell seeding before gelation, to cell seeding following gelation and granulation (figure 5.1). After initial experimentation, another modification of the gelation protocols was made, to prepare a 2% (w/v) hydrogel followed by hydration to a 1% (w/v) hydrogel using an equal total volume of media with the suspended cells (cell suspension). Concentration of the hydrogel before granulation allowed the addition of cells and media to the hydrogel construct while maintaining the intended hydration of the hydrogel. Previous experiments using the 1% (w/v) hydrogel resulted in overhydrated, low stiffness hydrogels due to the inevitable addition of liquid in the form of the cell suspension to the construct. These hydrogel scaffolds could not survive long term culture as they were too soft, and they disintegrated within 1 day. After hydrating the concentrated hydrogel using the cell suspension of equal volume, the hydrogels were of the appropriate hydration and stiffness. They remained intact for a longer time than the non-concentrated hydrogels, however they still did not remain intact for the full duration of the experiment.

The third protocol modification of adding 4% (v/v) post-granulation crosslinker was the solution to this issue. Adding the post-granulation crosslinker resulted in the formation of a hydrogel scaffold that remained intact for up to 28 days in culture, due to the formation of new crosslinked bonds between granules via Michael-type addition between the free thiol groups on the surface of the hydrogel granules and the acrylate groups on the added PEG-diacrylate crosslinker molecules. Modifying the protocol to prepare a more concentrated hydrogel, followed by further crosslinking of the granules using post-granulation crosslinker allowed addition of the cells to the granular scaffold without sacrificing the mechanical properties or the long term stability of the scaffold.

Finally, a modification was made incorporating growth factors into the hydrogel construct. Adding BDNF to the cell construct was shown to improve the cell survival in the hydrogel environment [22], [74], [75], [141]. BDNF works particularly well for the neural cells since this growth factor is present during development and maintenance of the nervous tissue, and therefore adding it to the hydrogel construct will allow the *in vitro* environment to better replicate the *in vivo* one. Addition of BDNF to the hydrogel scaffold allowed for improved neuron survival in the scaffold, and supplementation of the media with BDNF, followed by exchanging the media every two days, allowed refreshing of the BDNF in the scaffold environment for maintained cell viability and neurite formation within the scaffold.

These protocol modifications, informed by hydrogel characterization, allowed the production of the user modified granular hydrogel. The characterization, optimization, and cell seeding experiments comparing the granular hydrogel to the unmodified, non-granular hydrogel, as well as to the TCP control, are detailed in the following sections.

5.5. Scaffold Stability Characterization

The 28 day culture showed that the 4% (v/v) post-granulation crosslinker (PGC) provided sufficient stability for cell culture without affecting cell connectivity (figure 5.4). With 2% (v/v) PGC added to the granular hydrogels, cell culture resulted in constructs that could not remain intact over the experimental period. The granules of these hydrogels lacked sufficient crosslinks between them and therefore dispersed over time, resulting in scaffold degradation. At concentrations above 4% (v/v) PGC, while the scaffolds remained intact, there was visible restriction of movement of the cells. Cells in these hydrogels were observed organized in smaller, more dispersed, cell clusters as the PGC concentration increased (figure 5.4A). This was likely due to the increased stiffness of the hydrogel environment through the greater number of crosslinks being formed between the granules. The 4% (v/v) PGC hydrogels allowed maintenance of the hydrogel scaffold over time, with minimal granule dispersion and scaffold degradation, while also providing an environment favouring larger and more closely connected cell cultures.

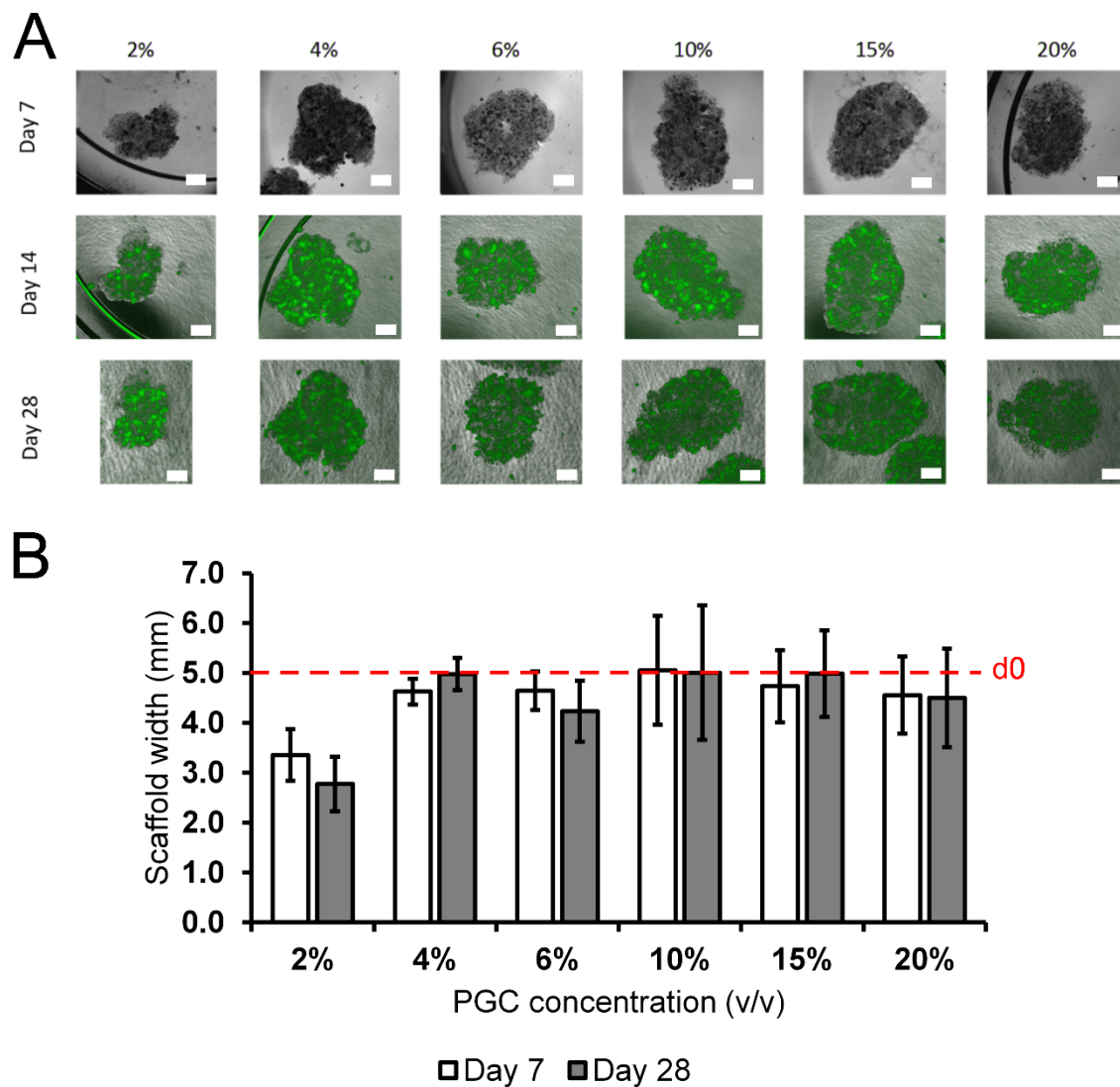


Figure 5.4. PGC optimization. **A.** Widefield images showing the size and cell distribution within the hydrogel scaffolds for the 6 investigated PGC (v/v) concentrations over the 28 day observation period. Scale bars = 1 mm. **B.** Column chart showing width analysis for day 7 and day 28. $N = 48$ width measurements. Approximate day 0 width was measured at 5 mm. Error bars represent standard deviation across measurements.

Post-granulation crosslinking could have additionally been monitored at the molecular level, using the Ellman's test to quantify and confirm that there are available thiol groups after granulation for reconnection of the granules to form a stable scaffold [142]. Additionally, rheological measurement of the scaffolds formed with varying amounts of PGC could give insight into the variation of hydrogel stiffness for comparison to the unmodified, non-granular hydrogel. However, for this portion of the project, considering the time constraints, a macro-scale visual observation was deemed sufficient to confirm scaffold maintenance over time, as well as to confirm the presence of available thiol groups given the assumption that there would be enough available thiol groups on the granules for the crosslinking reaction if the scaffold maintained the original size throughout the stability experiment.

5.6. Hydrogel Granule Size Characterization

Hydrogel granules were formed in non-uniform polyhedron shapes with approximately similar widths and lengths (figure 5.5D-F). Hydrogel granule diameters of granular hydrogels formed through the 40 μm , 17 μm , and 13 μm sieve sizes were calculated by taking an average of three different edge-to-edge distance measurements for each granule. Granule diameters were found to be 132 $\pm$ 70 μm , 124 $\pm$ 84 μm , and 99 $\pm$ 64 μm respectively (figure 5.5G-I). Compared to sieve size, granulation resulted in over 200% larger granule sizes for the 40 μm cell sieve and over 600% larger granule size for the 17 μm and 13 μm TEM grid sieve. This is probably due to two reasons: firstly, the compression and subsequent relaxation of the hydrogel as it moves under pressure through the mesh. For smaller sieve sizes, the hydrogel encountered a greater resistance and therefore underwent greater compression and dehydration before passing through the sieve. This resulted in formation of larger granule sizes relative to the sieve size [143], [144]. Secondly, the combination of granules with each other after they are formed on the other side of the mesh could have also affected the granule size. The higher pressures encountered by the hydrogel as it is extruded through the smaller sieve size mesh may have resulted in greater relative expansion and combination of granules to form larger than expected final granule diameters [145].

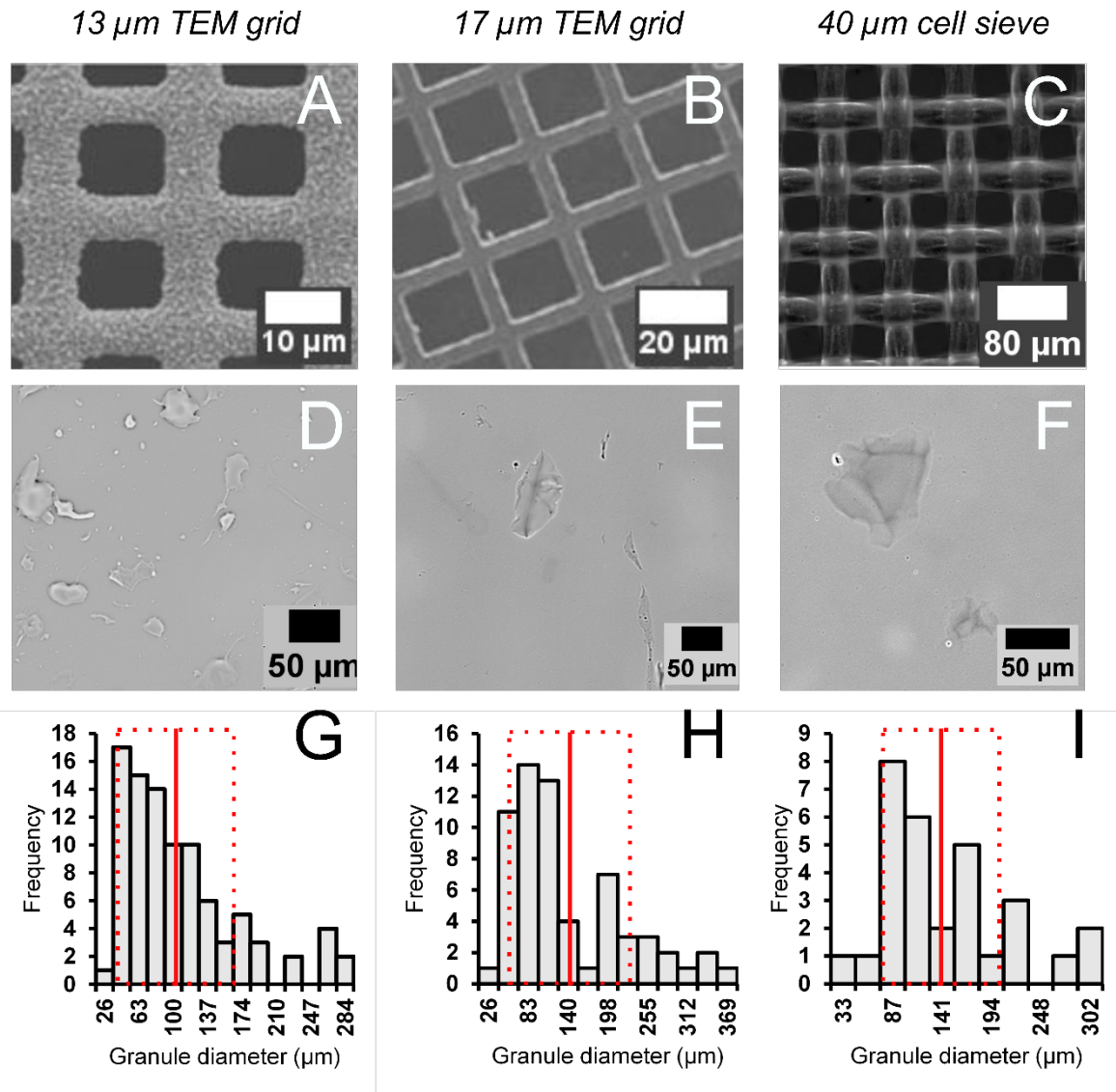


Figure 5.5. Granule size characterization. SEM images of the 13 μm TEM grid (A), scale bar = 10 μm , the 17 μm TEM grid (B), scale bar = 20 μm , and the 40 μm cell sieve (C), scale bar = 80 μm (A and B are adapted from Zhang et al. [146]). Widefield microscopy images of hydrogel granules formed from the 13 μm TEM grid (D), the 17 μm TEM grid (E), and the 40 μm cell sieve (F). Scale bars = 50 μm . Histograms showing distribution of granule diameter measurements for the 13 μm TEM grid (G), $N = 94$, the 17 μm TEM grid (H), $N = 63$, and the 40 μm cell sieve (I), $N = 30$. Red solid lines represent average granule diameter and red dotted boxes represent 1 SD.

Hydrogel granules were found to swell and increase in size after being placed in PBS (figure 5.6, supplementary figure S10). On average, granules swelled approximately 5.5 μm in diameter (figure 5.6E). Granules reached equilibrium after approximately 20 minutes, and maintained until observation was concluded at 100 minutes. As the hydrogel encountered an environment with high concentration of PBS, the solvent molecules penetrated into the hydrogel network, causing the granules to expand. Further penetration and swelling occurred until the elasticity force of the hydrogel boundary matched the opposing osmotic force of the PBS, preventing further deformation and establishing an equilibrium [147].

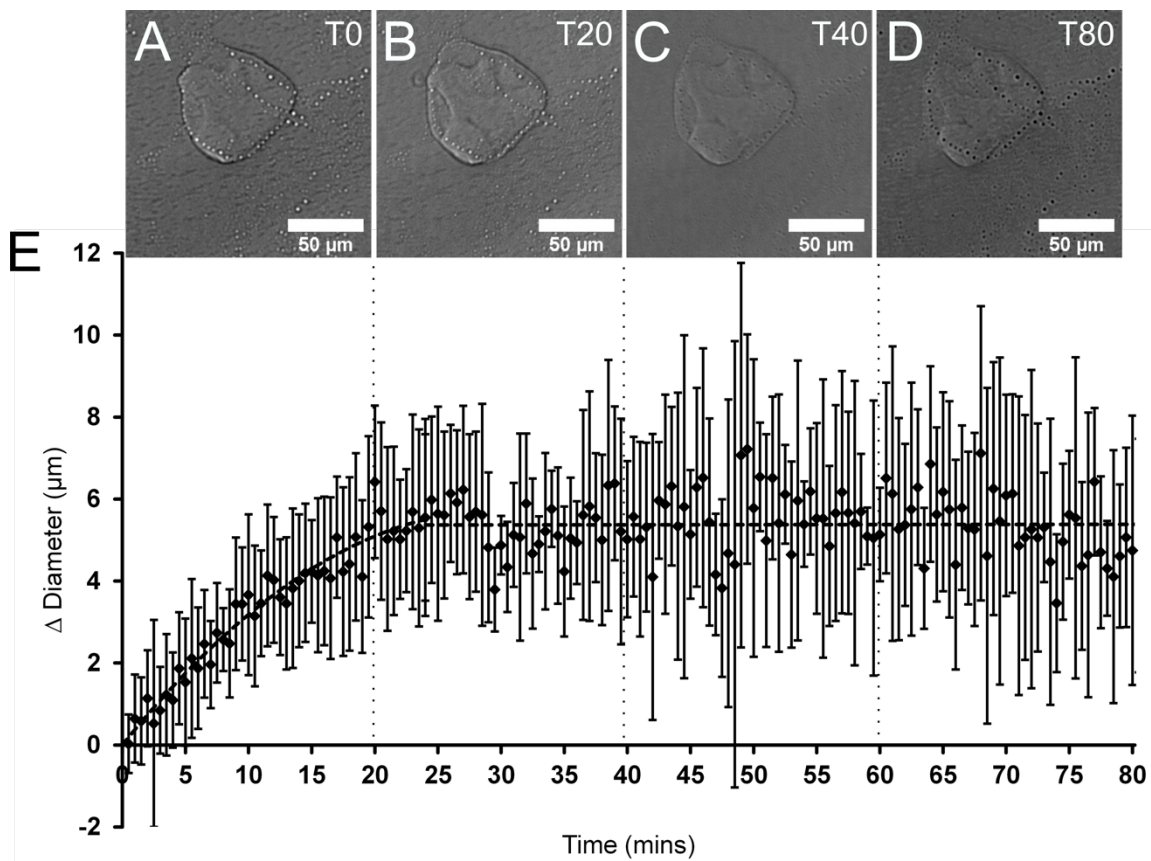


Figure 5.6. Hydrogel granule swelling. *A. – D.* Widefield images showing a hydrogel granule as it swells over time (mins). Scale bars = 50 μm . *E.* XY scatterplot showing the average change in diameter for the observed granule set. $N = 855$ diameter measurements. Error bars represent standard deviation across diameter measurements.

Neurons seeded within the granular hydrogels from three sieve sizes were all observed having extended morphology and forming connected networks. Over the 14 day experiment, cells in the control TCP culture were observed to have proliferated to gain approximately 15% in cell number between the day 7 and day 14 time points, while cells in the three granulation sizes of the granular hydrogels were observed gaining approximately 60% to 70% in cell number between the two time points (calculated from cell count data). The differentiated SH-SY5Y cells have reduced proliferative rate, explaining the low doubling time for the cells in culture. Moreover, the difference between the 2D TCP proliferation rate and the 3D granular hydrogel rates can be explained by the increase of surface availability in the 3D scaffold leading to an increase in proliferation.

Cell seeded granular hydrogels prepared from the three sieve sizes showed no significant differences in cell viability within the hydrogels (figure 5.7). Viability ranged from 52% to 77% (live cells/total cells) across groups, with the 17 μm sieve maintaining slightly higher viability than the other two groups, over the 14 day period. Given that the granules prepared from the three sieve size varied little in the diameter, results of similar cell viability within the hydrogels was expected.

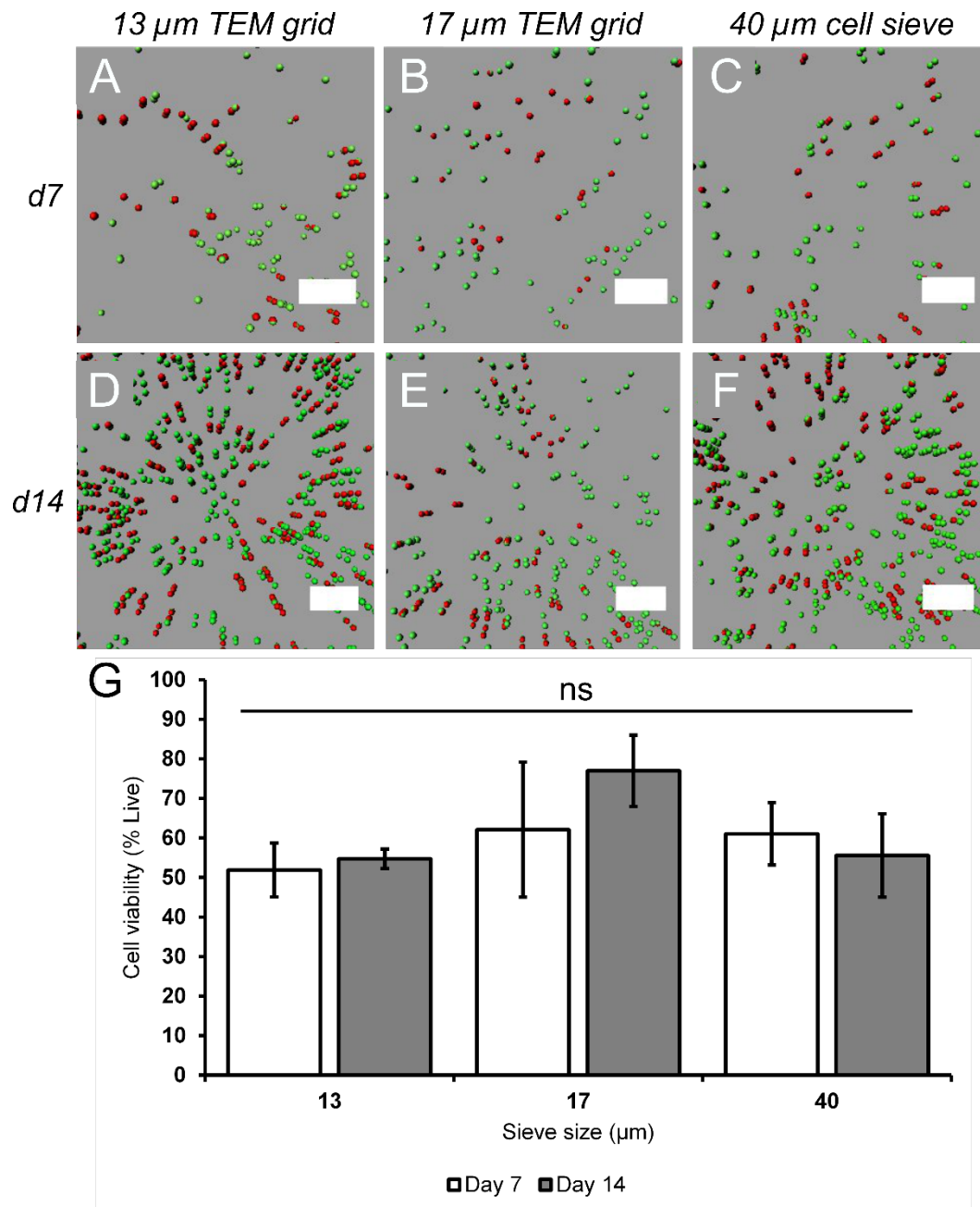


Figure 5.7. Cell viability for the three sieve sizes. Imaris 3D spot snapshots showing live-dead data analysis for the three sieve sizes at day 7 (**A-C**) and day 14 (**D-F**). Green spots represent live cells and red spots represent dead cells. Scale bars = 50 μm. **G.** Column chart showing cell viability for the cell seeded hydrogels from three sieve sizes. $N = 15$ viability measurements. ANOVA single factor test showed NS. Error bars represent standard deviation across viability measurements.

The ideal hydrogel granule size would be small enough to allow for attachment surfaces for the cells without hindering the cell-cell connections, and large enough to maintain hydration and diffusion to the centre of the hydrogel scaffold by creating sufficient hydrated space between the cells [148], [149]. This characterization experiment aimed to investigate whether a small difference in granule size would affect the environment's favourability to cells, and whether there was an optimal sieve selection of the three. Since the data showed that hydrogels prepared using the three sieves were of similar adequacy as a cell seeding environment with non-significant differences in viability across groups, it was pertinent to select the optimal sieve size based on efficiency of each granulation protocol.

The manipulation of the 40 μm sieve in practice was much more efficient and repeatable than that of the 17 μm and 13 μm TEM grids, due to the small size of the TEM grids requiring a more complicated procedure to set up the apparatus for granulation than for the cell sieve. For the 40 μm sieve, the syringe was simply filled with non-granular hydrogel, placed directly on top of the sieve, and depressed to granulate the gel. The cell sieve was large enough to remain in place and withstand the high pressures of granulation. For the TEM grids, a more technical procedure was required. The syringe was filled with non-granular hydrogel, and using a scalpel, a small incision was made in the syringe near to the exit point, and using tweezers, the TEM grids were inserted into the incision to hold the tiny grid in place so that it could withstand the pressures during granulation. Therefore, it was decided that the 40 μm sieve would be used for future experimentation.

5.7. Hydrogel Rheology Characterization

For viscoelastic materials such as hydrogels, the imposed stresses do not transfer directly to instantaneous deformation and resumption of the original shape upon removal of applied stress as happens during the elastic response. Instead, the applied stresses result in gradual deformation and recovery, accompanied by the dissipation of energy [150]. The combination of viscous and elastic response of the hydrogel allows for observation of a number of rheological properties.

The oscillatory shear rheology and DWS protocols were both used to collect preliminary storage and loss modulus measurements for gelation, static measurement, and swelling of the hydrogel, and it was found that the DWS protocol was more favourable for collecting the full rheology dataset. While the shear rheology method is more commonly used for rheological property measurement, the setup requires several samples for calibration. Values for normal force, oscillation frequency and amplitude needed to be determined through destructive testing. The 25 mm shearing plate size required a minimum 500 μl of hydrogel per sample, but in practice used around 1 ml of sample, and the preparation would become expensive when considering the high number of samples required for data collection using this device. On the other hand, the DWS is a novel rheological measurement device, however it uses a smaller, more exact volume per sample (400 μl) and due to the automated process, only required calibration of one property: the polystyrene bead concentration. This allowed for collection of the full dataset using a smaller, less expensive volume of hydrogel.

Additionally, the oscillatory shear rheology device produces a bulk property measurement giving one value of storage and loss modulus at each data collection point for the entire hydrogel sample. While this measurement is useful, it may not reflect the properties of the material on the scale of cellular interaction, which is important information to have when developing scaffolds for cell culture. Bulk and local rheological properties can differ for the

same material, and therefore a measurement of either may not represent the other. On the other hand, DWS provides an average of the local property values, giving an overall view of the hydrogel properties on a scale closer to the scale of cellular interaction.

There are other techniques that can be used to measure storage and loss moduli components of shear modulus, including steady-shear rheology which takes a simpler unidirectional plate movement for the measurement approach, and extensional rheology, which measures shear properties while elongating a cylindrical sample volume [151]. There are also other rheological properties, apart from the shear modulus response to shear stress, which could be measured for the hydrogel. These include the bulk (elastic) modulus response to uniform hydrostatic pressure, Young's modulus parallel response to uniaxial stress, and Poisson's ratio of orthogonal response to uniaxial stress [152], [153]. Shear storage modulus and loss modulus were chosen for the purposes of comparing measured values to known literature values for the chosen hydrogel and for the depth of information available from observing these values for the hydrogels. For reasons of machine availability, material cost, repeatability, and number of calibration values, it was decided to utilise DWS methods to measure the datasets of shear modulus components for hydrogel rheology.

Preliminary gelation investigation indicated that the minimal concentration for formation of non-granular hydrogels was likely between 0.5% and 1% (w/v), as the hydrogels made at 0.5% (w/v) concentration failed to gel, while hydrogel formation was possible for hydrogels made at 1% (w/v) concentration.

Preliminary time-lapse of gelation of the 1% (w/v) and 2% (w/v) non-granular hydrogels (NGG) using shear rheology indicated that after 30 minutes, the hydrogels obtained G' of 1 Pa and 3 Pa respectively (figure 5.8A, B). From this data, the 1% (w/v) hydrogel did not achieve gelation (determined by the achievement of approximate zero change of G' values) within 30 minutes, and the 2% (w/v) hydrogel obtained gelation within approximately 18 minutes. The G'' values were near zero throughout the entire measurement for both hydrogel groups, and therefore the gel point (the time at which G' increases dramatically compared with G'') could not be suitably estimated for the hydrogels using these measurements. These data were determined to be a false representation of the true rheology values due to the lack of normal force enforced in the equipment setup, to ensure contact with the hydrogel during data collection. Repeat gelation data and frequency sweep data were not collected.

In terms of diffusing wave spectroscopy, time-lapse of gelation indicated that after 30 minutes, the 1% (w/v) and 2% (w/v) NGG hydrogels obtained G' of 20 Pa and 190 Pa respectively (figure 5.8C, D). From this data, the 1% (w/v) and 2% (w/v) hydrogel obtained gelation within approximately 23 and 15 minutes respectively. The gel point was estimated at 8 and 2 minutes respectively.

Frequency sweeps of the 1% (w/v) and 2% (w/v) hydrogels indicate gel behaviour ($G' > G''$) over the majority of the investigated frequency range (figure 5.8E, F). Gel character remained for the 1% (w/v) hydrogel until approximately 10 kHz, while for the 2% (w/v) hydrogel the crossover point from gel character to liquid character extended to 11.5 kHz.

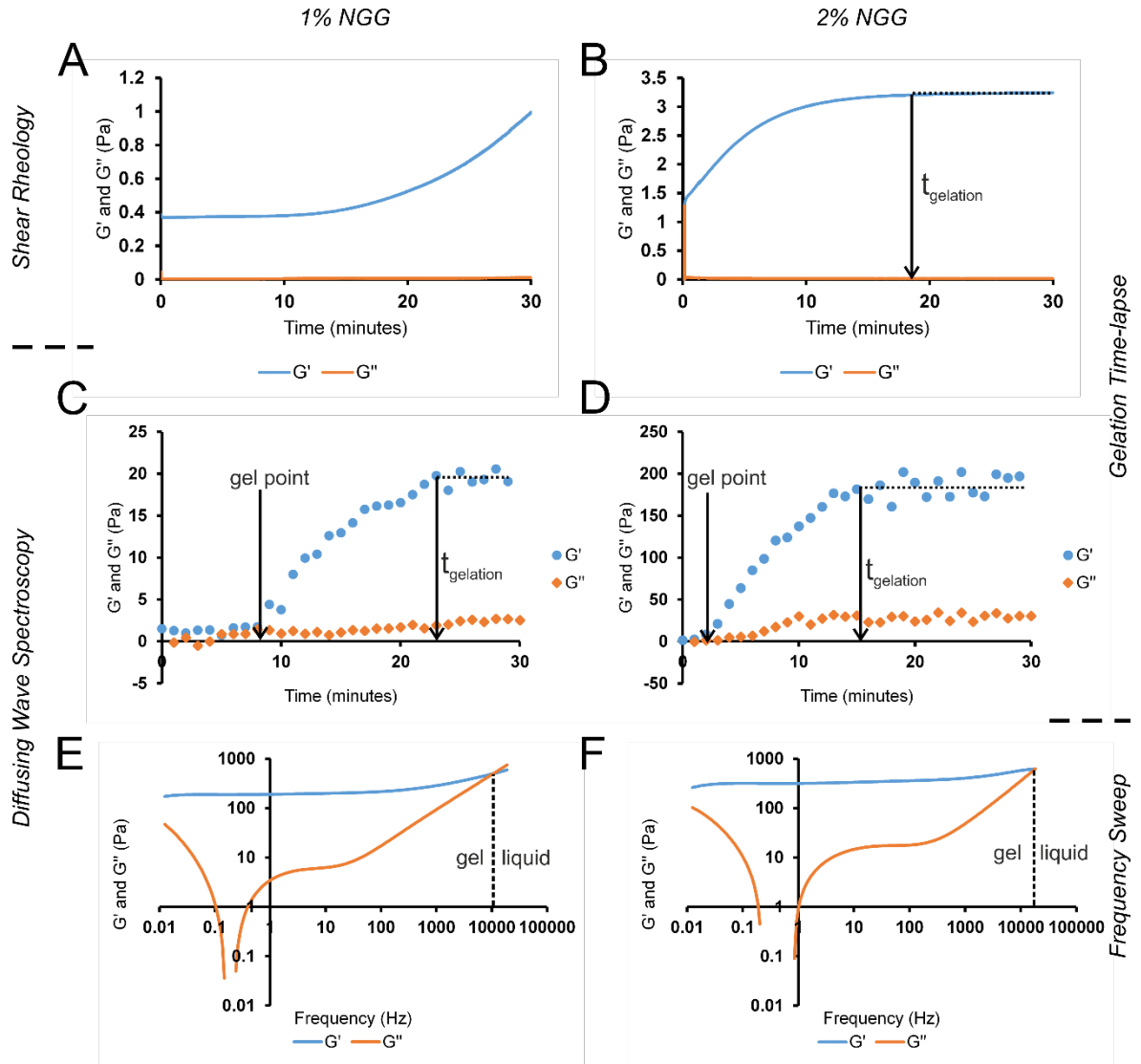


Figure 5.8. Comparison of Shear Rheology and Diffusing Wave Spectroscopy (DWS) techniques. XY scatterplots show change in storage modulus (G') and loss modulus (G'') for the 1% (w/v) NGG (**A**, **C**, **E**) and the 2% (w/v) NGG (**B**, **D**, **F**) during gelation using shear rheology time-lapse (**A**, **B**), during gelation using DWS time-lapse (**C**, **D**), both at 1 Hz frequency oscillation, and during frequency sweep using DWS at $t = 24$ hours (**E**, **F**). For **C** and **D**, values are a triplicate average, and error bars showing standard deviation have not been included, to emphasise data changes over time.

Additionally, it was found that the bulk values for 1% (w/v) and 2% (w/v) NGG were at G' 164 Pa and 309 Pa after 24 hours crosslinking. The granular hydrogels, prepared by granulating non-granular hydrogels of the same concentration (no media additions or dilutions) were slightly less viscous at 113 Pa and 276 Pa respectively (figure 5.9F). Although the difference is not statistically significant, it is likely that the granulation of the hydrogels indeed slightly reduced the hydrogel stiffness; mechanical fracturing of the hydrogel construct would break a number of crosslinked bonds, decreasing the mechanical stiffness in these areas of the hydrogel and reducing the measured rheological stiffness.

Swelling of the 2% (w/v) hydrogel by adding an equal volume of PBS and 4% (v/v) PGC showed an increase in G' of approximately 150 Pa over 120 minutes (figure 5.9G). This increase of stiffness was due to opposing rheological changes during swelling and post-granulation crosslinking. Hydration of the granules and dilution of the hyaluronic acid concentration would decrease G' , while post-granulation crosslinking would produce a secondary gelation-like effect, competing with the hydration and dilution to result in an overall increase the G' values over time.

Measurements from the DWS were determined to be a more accurate representation of true rheology values, since the recorded DWS measurements of G' and G'' matched the visually observed gelation time estimated during preliminary experimentation. Moreover, bulk stiffness of the hydrogels at 1% (w/v) and 2% (w/v) corroborated findings from Farrell *et al.* [154].

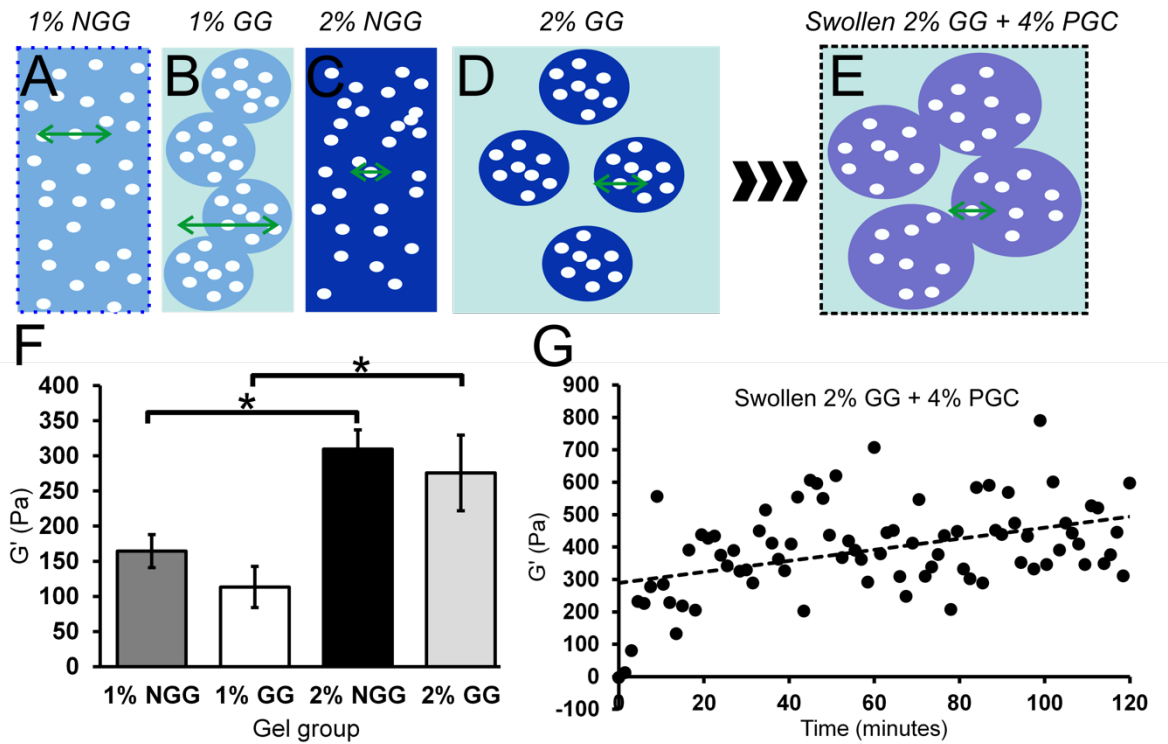


Figure 5.9. Rheological characterization of the hydrogels with the incorporated protocol modifications. Schematic drawings of the **A.** 1% (w/v) non-granular hydrogel (NGG), **B.** 1% (w/v) granular hydrogel (GG), **C.** 2% (w/v) NGG, **D.** 2% (w/v) GG, and **E.** swollen 2% (w/v) GG. White dots represent location of PS microbeads for light scattering measurement of stiffness using diffusing wave spectroscopy (DWS). Green arrows represent relative vibration of PS microbeads in the hydrogels. **F.** Column chart showing G' values for the four hydrogel groups. $N = 12$. Error bars represent standard deviation across measurements. * Bonferroni adjusted post-hoc student's t -test show $p < 0.013$ comparing the NGG to the GG for both 1% (w/v) and 2% (w/v) concentrations. **G.** XY scatterplot showing G' over time for the swelling of a 2% (w/v) granular hydrogel combined 1:1 (v/v) with PBS and 4% (v/v) PGC. $N = 81$.

5.8. Cell Viability

Live–dead sacrificial assays showed that the percentage of live cells in culture ranged from 43% to 71% (live cells/total cells) across all groups (figure 5.10G). Compared to the TCP control, the non-granular hydrogel had slightly lower cell viability measurements while the granular hydrogel had slightly higher cell viability measurements. However, given that there was no statistical difference across groups (ANOVA, NS) the data implies that the viability is comparable across the three groups, indicating that the hydrogel environment provides a living environment of comparable to the control.

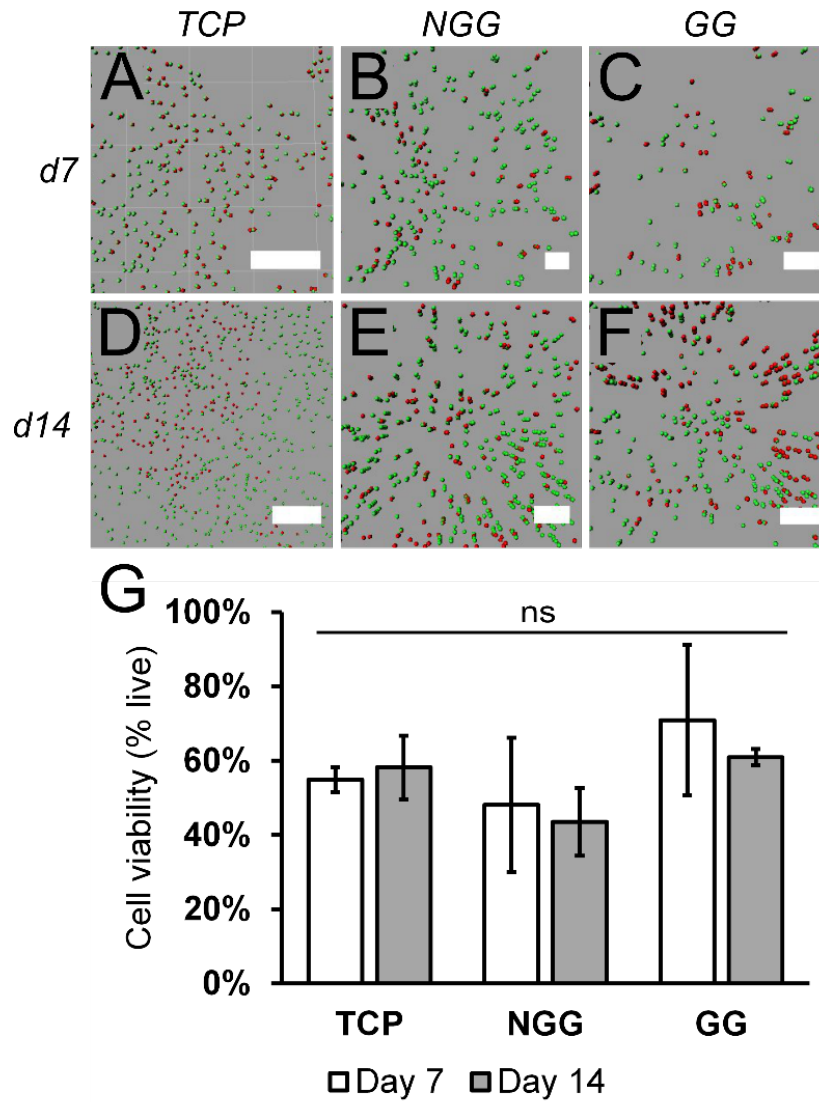


Figure 5.10. Cell viability for the three groups. Imaris 3D spot snapshots showing live-dead data analysis for the TCP, NGG, and NG groups at day 7 (A-C) and day 14 (D-F). TCP = tissue culture plastic, NGG = non-granular hydrogel, GG = granular hydrogel. Green spots represent live cells and red spots represent dead cells. Scale bars = 50 μ m. **G.** Column chart showing cell viability for the cell seeded hydrogels from the TCP, NGG, and NG groups. $N = 15$ viability measurements. ANOVA single factor test showed NS. Error bars represent standard deviation across viability measurements.

5.9. Live Cell Imaging and Analysis: Neurite Growth and Connectivity

Qualitative analysis of the live images show a number of differences comparing the control culture, the non-granular hydrogel culture, and the granular hydrogel culture (figure 5.11, supplementary figures S11-13). By day 7, 2D TCP control cultures contained neurons with highly confluent connected cultures (figure 5.11A, D). Cells were distributed across the surface, forming robustly connected networks with elongated morphology and extended neurites. The neurons were observed exploring the nearby environment for short distances, where movement was restricted by limited space between cells and also by the neurite connections between them.

At the same time point, non-granular hydrogels contained neurons distributed into spherical clusters (figure 5.11B, E). Clusters contained cells with rounded morphology; the majority of cells did not have extended neurites. The clusters of cells were not connected to each other, and were approximately 20-100 μm in diameter. Neurons were observed remaining static, without exploring the environment except for the extension of neurites from a small number of cells positioned on the exterior of the cell clusters.

Granular hydrogels contained neurons also distributed into clusters as with the non-granular hydrogels, however the clusters were generally closer to each other, less distinct from each other, and more interconnected with each other than those in the non-granular hydrogels (figure 5.11B, C, E, and F). Clusters contained cells with elongated morphology with extended neurites, more similar to the 2D TCP control cells than to those in the non-granular hydrogels. Neurons in the granular hydrogels were observed exploring the nearby environment for short distances, where movement was restricted by the neurite connections between cells and clusters.

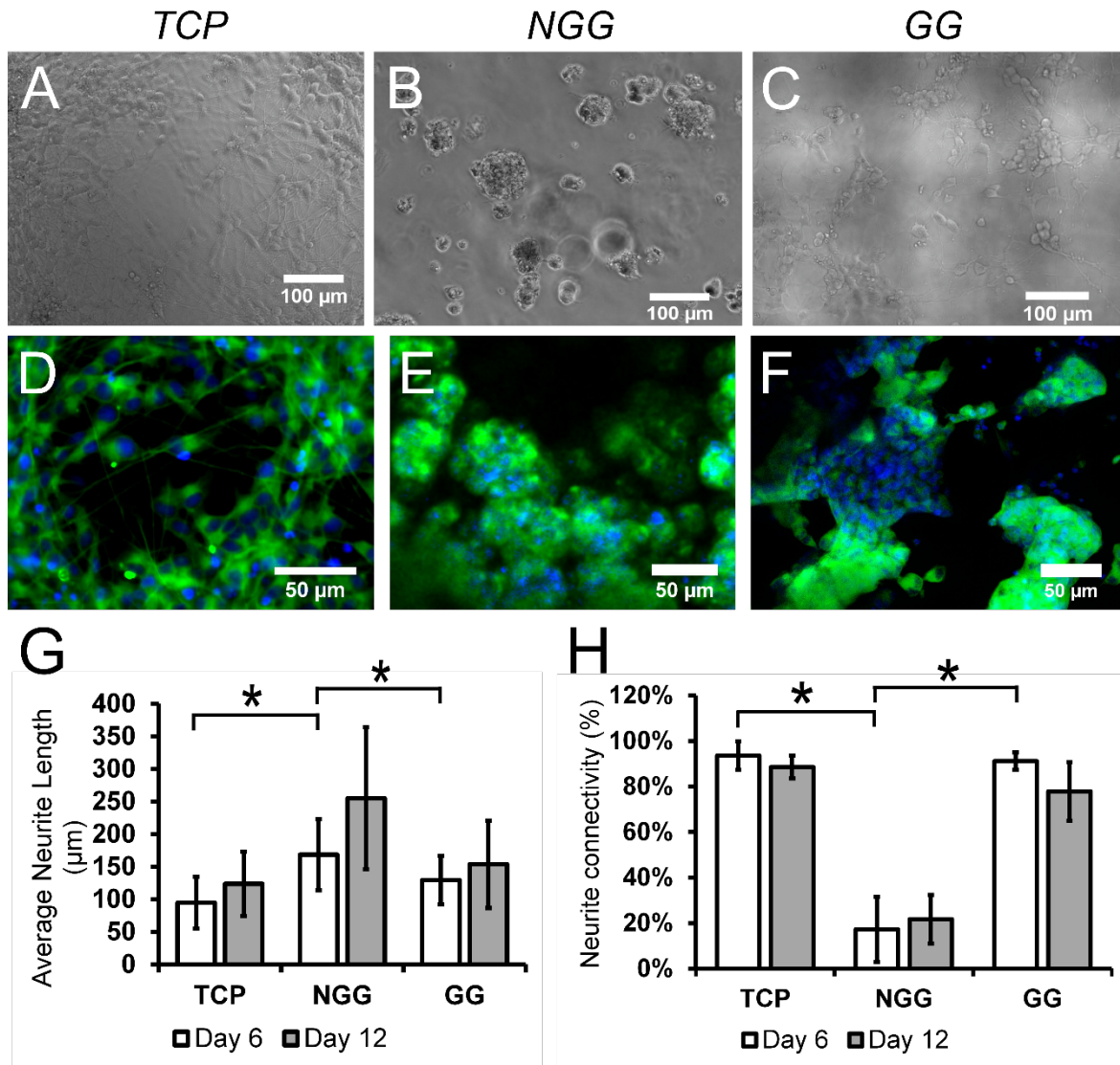


Figure 5.11. Live imaging characterization of the three groups. Day 12 widefield (A-C) and day 7 confocal slice sum Z-projection (D-F) images showing the cell morphology in A, D. the TCP 2D control, B, E. the non-granular hydrogel (NGG), and C, F. the granular hydrogel (NGG). Scale bars = 100 μm (A-C) and 50 μm (D-F). Column charts showing G. Average neurite length ($N = 420$ length measurements), and H. Neurite connectivity ($N = 1078$ cell observations), for the three cell seeding groups. ANOVA tests show $p < 0.05$. * Bonferroni adjusted post-hoc student's t -test show $p < 0.017$ comparing the TCP to non-granular hydrogels and granular hydrogels. Error bars represent standard deviation across measurements.

Quantitative analysis supported the qualitative indication of differences between the TCP, NGG, and GG cultures. To observe neurite length in the cultures, the total lengths of 70 neurites in the field of view from cell body to tip were measured and averaged using ImageJ software for each group, and quantitative analysis of the images taken at day 6 versus day 12 showed that, over the two time points, there was an increase in average neurite length for all groups (figure 5.11G). Comparing neurite lengths between groups, it was seen that the granular hydrogels produced an average neurite length comparable to the controls while the non-granular hydrogels produced cultures with increased neurite length. This can be explained by the observed cell distribution in the three networks. The control and granular networks were highly connected, with cells extending neurites to meet neighbouring cells in close proximity. The non-granular hydrogels were not connected, and the few neurons that were extending neurites into the surrounding stiff hydrogel environment were doing so in an attempt to meet neighbouring cells a great distance away. Therefore, the neurite lengths were increased for the non-granular hydrogels, while the neurite lengths were more similar to the control for the granular hydrogels.

To observe the neuron connectivity in the cultures, the neurons with neurites that made a connection with at least one other neuron in view (neurites visibly touching) were marked and counted using ImageJ software. This number was compared with the count of the total number of neurons for each group to give a percentage of connected neurites. Data regarding the percentage of connected neurites showed a range from 17.2% to 93.6% connectivity over all groups (figure 5.11H). Analysis across groups showed that the granular hydrogels had a connectivity greater and more similar in value to the TCP control when compared with the non-granular hydrogels, and this was true for both time points. Again, morphological analysis of the live images confirmed the differences in connectivity. Since the non-granular hydrogels contained a majority of neurons which were growing in rounded clusters and not

extending neurites, while the granular hydrogels contained a majority of neurons which were growing with elongated shape and extending neurites to form connections with neurons nearby, the connectivity was improved for the granular hydrogels.

To observe the real time neurite extension and retraction in the cultures, the change in total length of neurites over time was measured using time-lapse imaging of the neurons in each group at each time point. The length of 10 neurites in the field of view was measured every 10 minutes for 60 minutes and analysis of the average change in length indicated differences in the motility of neurites in the TCP, NGG, and GG cultures (figure 5.12). For the TCP cultures, neurites were extending and retracting over a maximum distance of 39 μm length compared with 46 μm and 60 μm for the GG and NGG cultures respectively. The more gradual extension of the neurites in the TCP and GG cultures, compared with the more drastic extension in the NGG cultures might again be explained by the arrangement of neurons and the ability of the cultures to form connected networks. Neurites in the TCP and GG cultures would not have to travel great distances to interact with and form connections with neighbouring neurites. Neurons in the NGG cultures which managed to overcome the stiff environment would extend neurites that would need to extend over much longer distances before making connections with neighbouring neurites.

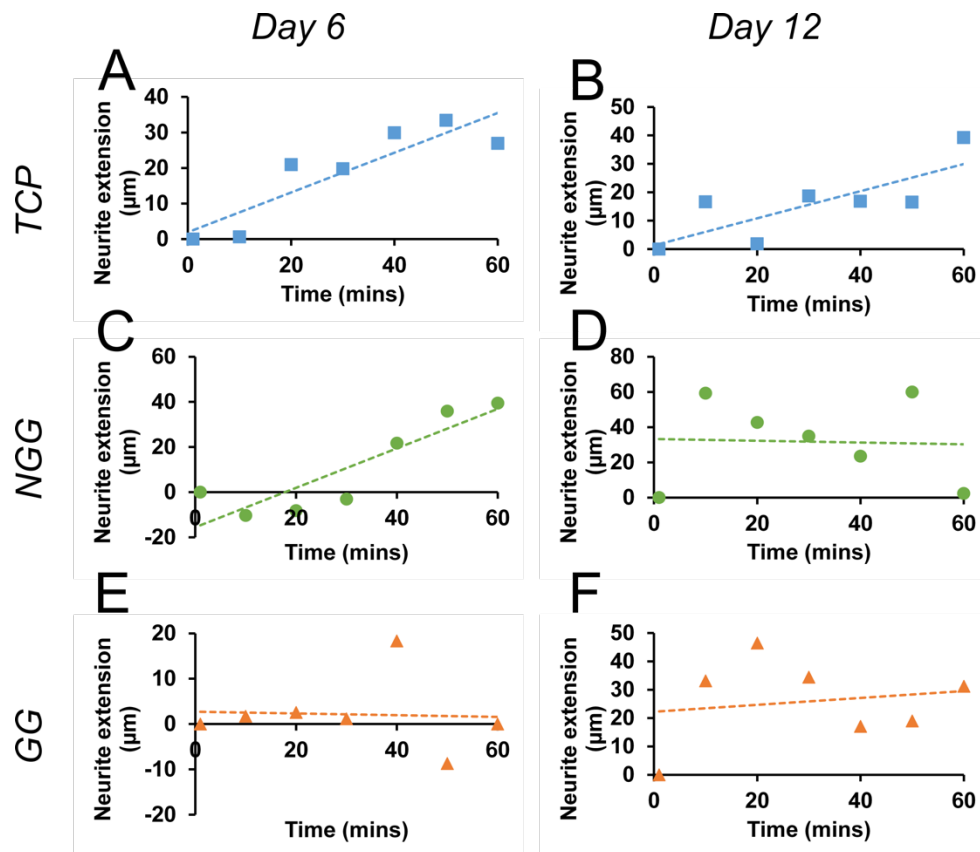


Figure 5.12. Quantitative analysis of the neurite extension in the three groups. Scatter plots show average neurite extension against time for the TCP (**A, B**) the NGG (**C, D**) and the GG (**E, F**) at day 6 (**A, C, E**) and day 12 (**B, D, F**). $N = 70$ spatial neurite measurements. Error bars showing standard deviation have not been included, to emphasise data changes over time.

5.11. Comparison of the TCP, GG, and NGG

The live cell imaging experiments demonstrated that the granular hydrogel was an improvement over the non-granular hydrogel for neural cell culture. While the non-granular hydrogels performed similarly to the granular hydrogels in terms of cell viability, they did not perform as well in terms of mimicking neurite length and neurite connectivity present in control cultures. There was an increase in neurite extension and average neurite length as well as a decrease in percentage neurite connectivity, comparing the non-granular hydrogel to the TCP control. This is probably due to the restrictive effect of the hydrogel in its non-granular state. The stiff environment created when the hyaluronic acid chains are fully crosslinked, prevents abundant growth and connection of neurites from neighbouring neurons. Studies have shown that neural cultures are affected by the mechanical properties of the scaffold environment and they prefer environments with stiffness comparable to native neural tissue (approximately 100 Pa) [155], [156]. Therefore, while there were a number of neurons that were able to extend neurites into the non-granular hydrogel, they were few and far between. This scarcity of neurites in the non-granular hydrogels led to longer average neurite lengths as the few neurites present extended further to make connections than in the control and the granular hydrogels.

On the other hand, in the granular hydrogels, the neurite connectivity over the time period was comparable to the control cultures. The granulation process allowed seeded neurons to move freely within the matrix and extend neurites to form interconnected networks. In other investigations, chemical modifications have been made to the hydrogel construct in order to create the microgels for cell seeding. Kuo *et al.* used a hybrid hyaluronic acid and gelatin construct crosslinked with methacrylic anhydride, and through a lengthy freeze drying and droplet forming photocrosslinking process, prepared individual microgel particles of about 150 μm to 300 μm [157]. Du *et al.* also used the bottom up approach, utilizing

photolithography to prepare intricate ‘lock and key’ shapes of polyethylene glycol methacrylate particles to build the hydrogel scaffold [158]. The investigated granulation process follows a similar motivation of building a microgel scaffold for improved cell culture, however uses a much simpler and quicker top-down approach by physically breaking the bulk hydrogel into smaller particles rather than building up from small particles to form the hydrogel scaffold. In this way, the robust modified hydrogel preparation and cell seeding protocol generates an improved three-dimensional scaffold mimicking ECM and providing high cell viability, and network connectivity.

5.12. Chapter Conclusion

This chapter investigated the influence of scaffold structure on the neural network formation in three-dimensional hydrogel scaffolds. For non-granular HA hydrogels prepared according to kit specifications, the neural networks consisted of rounded neurons, and few of the neurons extended neurites, explored the surrounding environment, and made connections with neighbouring cells. The rheological structure of the hydrogel was manipulated to develop a granular hydrogel with areas of reduced stiffness and rich ECM, which would allow cellular elongation and neurite extension of neurons, and form more connected neural networks within the hydrogel scaffold.

Modifications to the hyaluronic acid gelation process were optimized for simplicity of granule preparation using a 40 μm cell sieve followed by 4% (v/v) PGC to maintain stability of the granular hydrogel scaffold. Rheological characterization of the non-granular and granular hydrogels indicated how the hydrogel stiffness was affected by protocol modifications. Cell seeding experiments in the granular and non-granular hydrogels showed that granulation improved the cell morphology, neurite extension, and neural network

formation, resulting in cultures more similar to the control than within the non-granular hydrogel, and maintaining a cell viability comparable to the control as well.

The modified protocol resulted in development of a 3D culture scaffold for *in vitro* neuron cultures mimicking cell viability and connectivity of an *in vivo* environment. Moreover, it used a straightforward and simple top-down approach for granule preparation, and the hydrogel scaffolds are suitable for long term culture. These findings indicate that there is strong potential for the application of this protocol to a plethora of *in vitro* 3D human neural models, such as human “brain on a bench” relevant for drug testing or disease constructs, or medical technologies, such as artificial peripheral nerve grafts.

Chapter 6: Extruded Patterning of Granular Hydrogel Scaffolds for Human Neural Network Culture

6.1. Chapter Introduction

In the previous chapter, neural growth in a hydrogel scaffold was facilitated through the use of a granular hydrogel. Within this approach, the crosslinked hydrogel formed the primary structure of the scaffold and forced extrusion was used to break this hydrogel into a random granular secondary structure similar to the cellular architecture of neural tissues. However, tissues are not random in structure and have a high degree of organization at all length scales (figure 6.1A). This chapter explores the possibility of creating a tertiary ordered structure through use of a further extrusion step. A 3D printed device is used to produce a streamed flow of granular hydrogel with a multi-core layout (figure 6.1C). Crush and tear injuries can lead to the generation of large gaps in peripheral nerves and the ability to generate tertiary structured implants is a prerequisite for generating improved nerve guide conduits suitable for use in nerve gap repair.

Two versions of the tertiary (3°) extrusion device were designed and characterized using fluid modelling and hydrogel extrusion to inform selection of the optimal version for cell seeding experimentation. Version 2 of the device produced hydrogels with increased interspersions of the cellular and acellular hydrogel areas compared with version 1 hydrogels, and was selected as the more optimal device. Cell seeding experiments comparing the tertiary extruded hydrogels with non-tertiary extruded hydrogels showed increased preference for cells to remain deposited within the cellular hydrogel areas of tertiary extruded hydrogels over those of non-tertiary extruded hydrogels. Moreover, electrical activity was increased for the networks within the tertiary extruded hydrogels compared with the non-tertiary extruded hydrogels. Cell alignment was not improved with the addition of tertiary structure to the hydrogel networks, however, there is potential for addition of further directional cues to the scaffold to achieve alignment on the single cell scale, advancing this protocol as suitable for application toward improved artificial nerve graft technology.

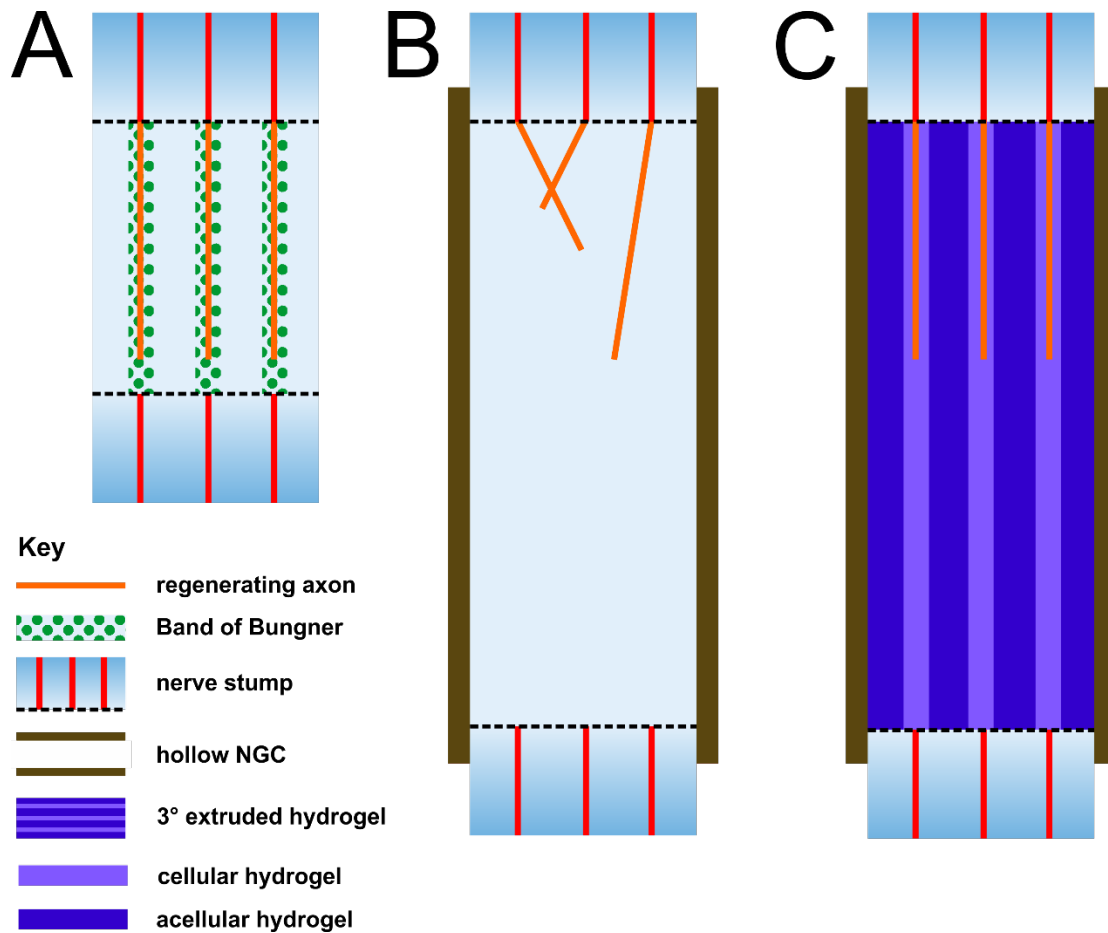


Figure 6.1. Schematics of section view of the nerve showing native regeneration over short gap distances (< 4 mm) (A), regeneration assisted by a hollow artificial nerve graft conduit (NGC) over longer gap distance (> 30 mm) (B), and proposed improvement to the NGC, adding the tertiary (3°) extruded hydrogel to direct regeneration over the longer gap distances (> 30 mm) (C). The tertiary extruded hydrogel is constituted of a cellular granular hydrogel of lower stiffness and fortified with BDNF, and an acellular granular hydrogel with higher stiffness and not fortified with BDNF.

6.2. Tertiary Extrusion Device Design and Printing

Bioprinting of artificial tissues has developed over the last decade as a method of preparing multi-layer, three-dimensional tissue models *in vitro*, and extrusion based devices have recently been adapted for use with biological materials [159]–[161]. Luer-lock devices have previously been used in conjunction with automated or manual syringe pumps, however the design of a multi-luer-lock device adapted for heterogeneous biological scaffold engineering, such as the tertiary extrusion device investigated in this project, has not yet been reported [162], [163].

The tertiary extrusion device was designed to prepare an ordered structure of alternating cell-seeded and acellular hydrogel strips, which would mimic the extracellular matrix during nerve regeneration *in vivo*, and direct cell distribution and longitudinal alignment. The optimal extrusion device would result in a hydrogel scaffold with cellular hydrogel columns interspersed in, and surrounded by, acellular hydrogel (figure 6.1C). This hydrogel scaffold, with granular secondary structure and ordered tertiary structure, should allow cells to be distributed and aligned longitudinally within the cellular hydrogel areas, mimicking cell distribution and alignment within a damaged, regenerating, peripheral nerve [124], [164].

Version 1 of the tertiary extrusion device was designed with two inputs merging into one output, through which the inputs would flow to form alternating cores (figure 6.2 v1). The input sections consisted of shrouded female luer-locks placed parallel to each other, from which curved cylindrical paths were used to transport granular hydrogel with and without embedded cells. The 3 mm diameter cylindrical path, used for granular hydrogel without cells, was directed into 5 secondary cylindrical paths of 0.5 mm diameter, 1 situated centrally and 4 situated axially around the central point. The 1.4 mm diameter path, carrying cells and granular hydrogel, fed 5 secondary cylindrical 0.5 mm paths that merged into a single flow in the output of the system.

Version 2 of the device adapted from version 1, has an additional diffuser plate that distributes the flow of the cell containing hydrogel across the output flow (figure 6.2 v2). To achieve this, the 1.4 mm diameter cylindrical path was directed into 8 secondary cylindrical paths of 0.3 mm diameter, situated in pairs, placed axially around the central point, alternating with the 4 axial secondary cylindrical paths described previously. The 13 secondary cylindrical paths emptied into one path as the output of the system.

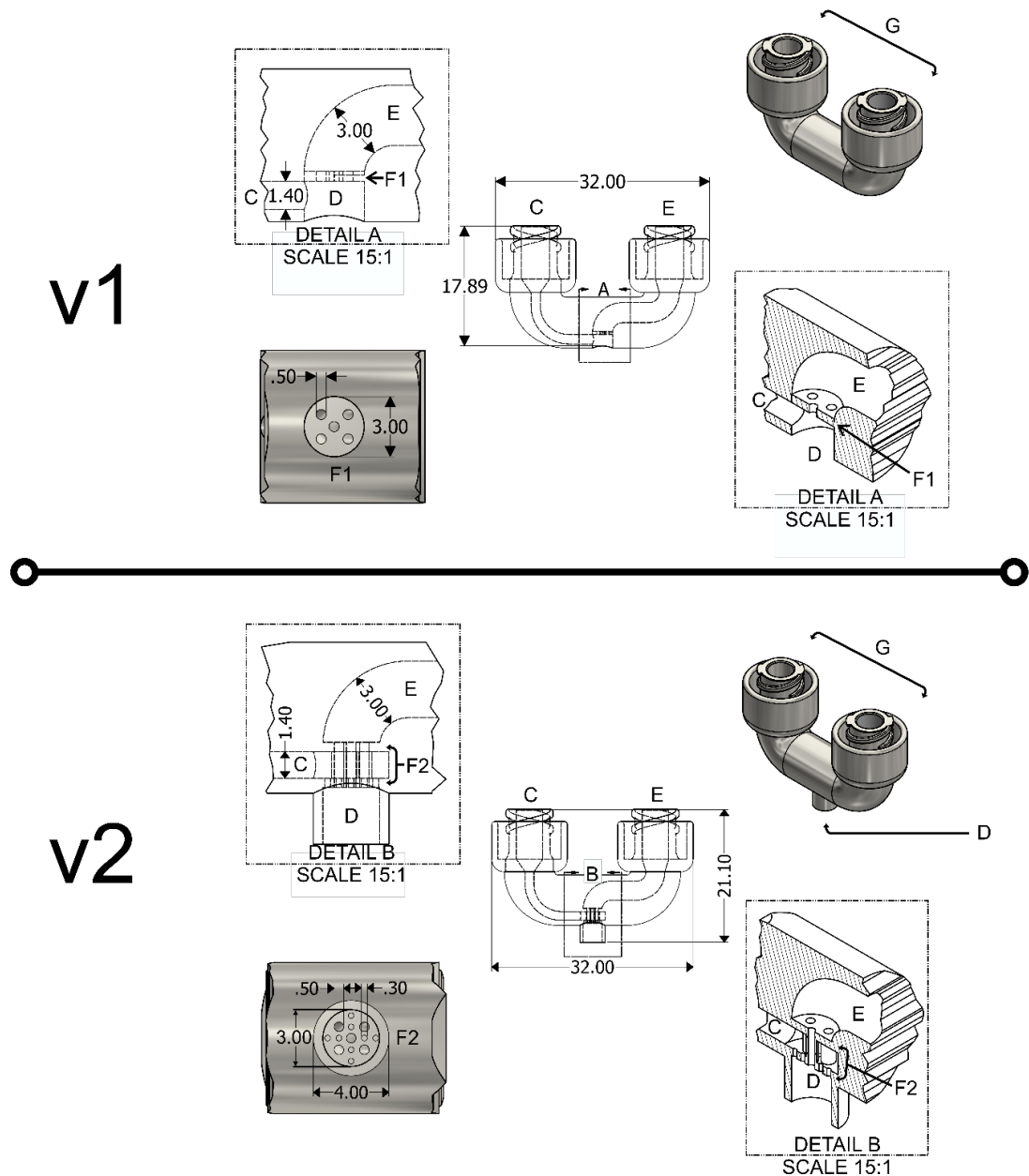


Figure 6.2. Schematic of versions 1 and 2 (v1, v2) of the device design. Cut-out detail views show meeting point of inputs and output for v1 (A) and v2 (B). C. Cellular granular hydrogel input. D. Output. E. Acellular granular hydrogel input. F1. Views of the 0.5 mm secondary cylindrical ports in 1 diffuser plate feeding the output for v1. F2. Views of the secondary cylindrical ports across two diffuser plates feeding the output for v2. In F2, two different diameters are used for the secondary cylindrical ports: 0.5 mm diameter for the acellular hydrogel output, and 0.3 mm diameter for the cellular hydrogel output. G. Shrouded female luer-locks functioning as input ports. Measurements are given in millimetres (mm).

Given that the *in vivo* nerve regeneration response involves physical alignment of Schwann cells to direct the regrowth of axons [44], this knowledge was used to inform design of the 3° extrusion device. The acellular 2% (w/v) granular hydrogel areas should have lower cell infiltration into them compared with the cellular granular hydrogel areas. Not only should the high stiffness conditions reduce cell migration into these areas of the hydrogel, but the absence of a rich laminin ECM and concentrated BDNF from these areas of the hydrogel, juxtaposed with the high chemoattractive cellular hydrogel, should also provide chemorepellent cues for migration of cells in the acellular hydrogel areas. In similar reasoning, the cellular hydrogel, fortified with a rich laminin matrix, concentrated BDNF, and with lower stiffness due to the 1:1 dilution with media, should provide a more favourable physical and chemical environment to maintain culture and migration within these areas of the hydrogel scaffold. Furthermore, in terms of unidirectional alignment of the cell neurites in the hydrogel, the gross structure should provide feedback of physical profile as well as chemoattractive gradient throughout the hydrogel [165], [166], resulting in directed neurite exploration and subsequent growth parallel to the alignment cue direction.

The 3° extrusion device was also designed with considerations made for the channel sizes to correspond to the stiffness of the input materials. While the acellular hydrogel channel would contain 2% (w/v) acellular granular hydrogel, the cellular hydrogel channel would contain effectively 1% (w/v) granular hydrogel, given the 1:1 dilution of the hydrogel with cells in media. The narrower diameter channel would allow for the less viscous 1% (w/v) granular cellular hydrogel mixture to be maintained under slightly higher pressure than the 2% (w/v) acellular granular hydrogel, allowing the extrusion of the combined hydrogels to occur at the appropriate mixing ratio [167], [168]. This is also the reasoning behind the 0.3 mm extrusion through the 8 secondary cylinders for the 1% (w/v) cellular hydrogel

compared with the 0.5 mm extrusion through the 5 secondary cylinders for the 2% (w/v) acellular hydrogel.

Printing resins used in cell culture need to support sterilization processes such as autoclaving. Durable resin was chosen for printing in order to allow the device to withstand the high temperature sterilization required for preparation of the material for cell seeding (121°C). However, this resin had a lower maximum attainable printing resolution than the standard resin, likely due to the specific combination of acrylate and methacrylate monomers and oligomers in the durable resin, and the requirement for a photo-initiated post-print curing process [169], [170]. This meant that some issues arose in printing the smaller cylindrical features of the device, and specific printing orientations were required to achieve devices with unclogged ports. The printing process involves photo-curing resin layer by layer, and since there was an intricate juxtaposition of the secondary cylinders in the output area, the device needed to be oriented with the ports at an angle that would allow the release of uncured resin from each layer before preparation of the subsequent layer (figure 6.3).

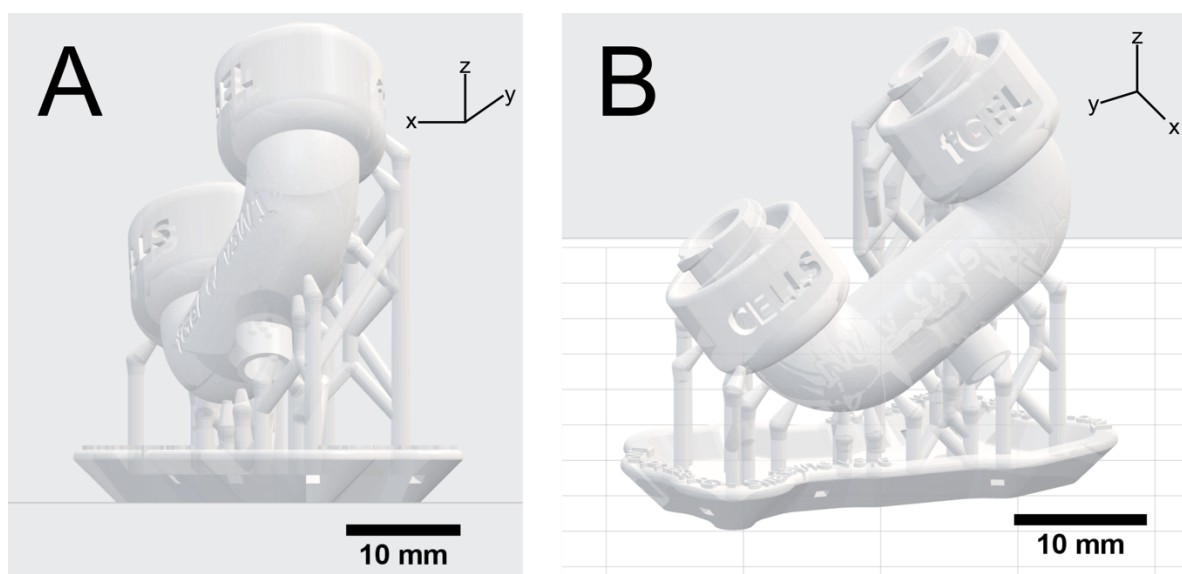


Figure 6.3. Screenshot images of the device orientation setup for printing using the Preform software showing **A.** 20° rotation on the x-plane and **B.** 40° rotation on the y-plane. Scale bars = 10 mm.

6.3. Fluid Modelling

ANSYS software fluid modelling showed the distribution of flow in the described conditions. For version 1 of the device design, modelling the flow path-lines indicated that the acellular granular hydrogel should be distributed into the 5 secondary cylinders and combine to one bulk flow upon exit from the cylinders. This bulk flow would be confined between the centre and the wall of the output opposite the cellular hydrogel input (figure 6.4B). The flow modelling demonstrated that the path-lines emerging from the cellular granular hydrogel input should not distribute or mix with the path-lines emerging from the 5 secondary cylinders, and instead remain as combined flow situated next to the wall of the output directly below the cellular hydrogel input point (figure 6.4A).

For version 2 of the device design, modelling the flow path-lines indicated that the acellular granular hydrogel input flow should distribute equally between the 5 secondary cylinders and remain distributed in this pattern at and beyond the output exit point (figure 6.4C). In the model, the path-lines emerging from the cellular granular hydrogel input should travel across the top of the cylindrical openings before progressing into the cellular secondary cylinders, to mix and become distributed with the path-lines from the 5 acellular secondary cylinders. The distribution should follow that of the radially alternating cellular and acellular granular hydrogel secondary cylinder distribution, and the path-lines should maintain this distribution pattern at and beyond the output exit point (figure 6.4D).

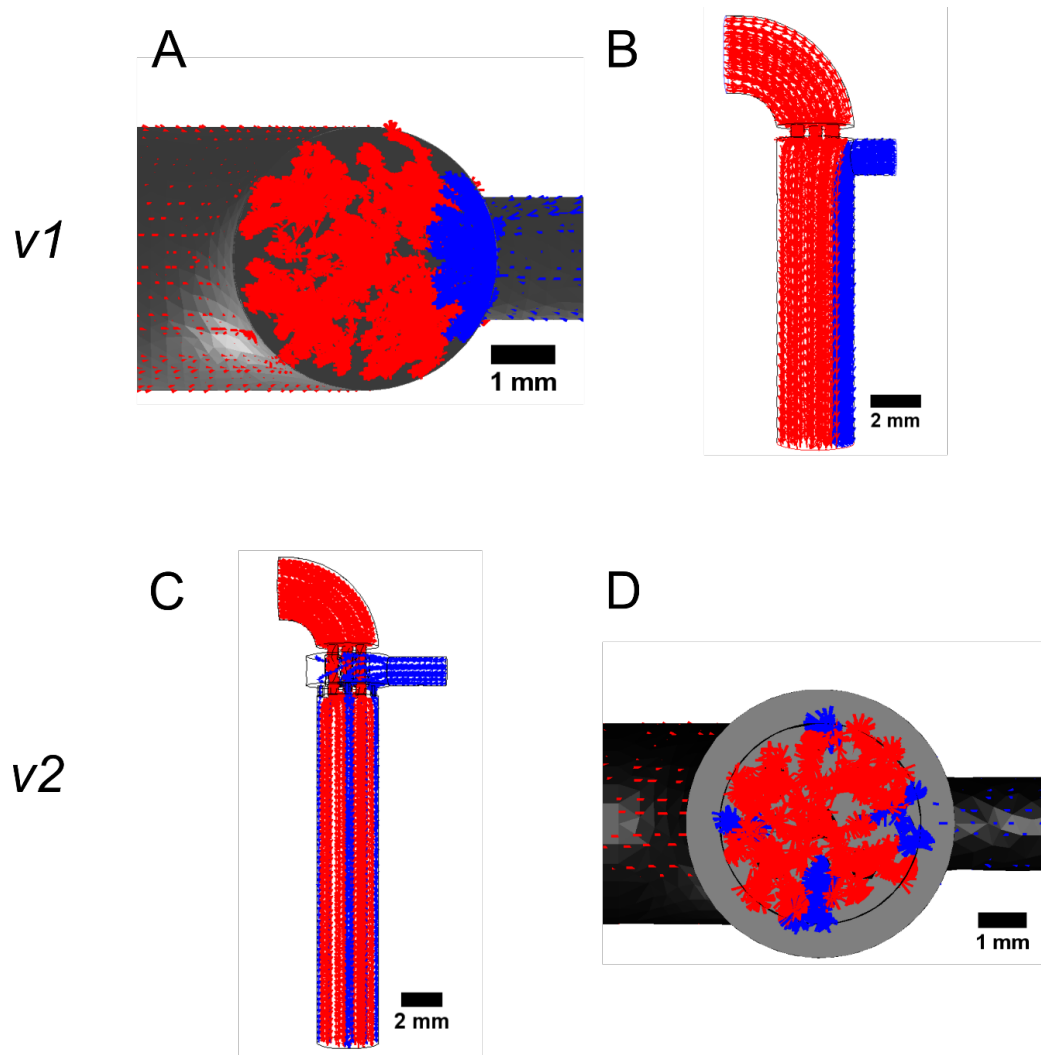


Figure 6.4. Graphical representation showing end-view (**A**, **D**) and side-view (**B**, **C**) of modelling results for v1 (**A**, **B**) and v2 (**C**, **D**) of the device design. Line-arrows from the acellular (red) and cellular (blue) inputs track particles from the inputs, through the secondary cylinders, and out of and extending beyond the output. Scale bars = 1 mm (**A**, **D**) and 2 mm (**B**, **C**).

The fluid modelling conditions were selected to demonstrate a viscous fluid flow. The acellular and cell-laden granular hydrogels both fluidize under compression, and have complex viscosity properties, however to decrease complexity of the fluid model, the hydrogels were modelled as a singular, highly viscous fluid. Fluid properties of density and viscosity were selected from collected rheological data (Chapter 5) and reported hydrogel densities. Viscosity of the acellular hydrogel (2% (w/v) GG) was measured at 42.6 kg/ms and that of the cellular hydrogel (1% (w/v) GG) was measured at 17.1 kg/m.s. The model observed a fluid with a viscosity at the average of the two measured values: 30 kg/m.s, and a density of 1140 kg/m³ as reported by Métrailler [171]. This simplified the granular hydrogel mechanical properties to one representative value to fit the fluid modelling software requirements and provide comparable, although not exact, solutions for the hydrogel motion in the device. The input speed was also selected as an estimated value related to the pressing down on the syringes: 1 cm/s, while the no-slip wall-shear condition and the zero output gauge pressure were selected to represent the normal conditions of the device being used at room temperature and pressure [172].

6.4. Hydrogel 3° Extrusion Characterization with Fluorospheres

Acellular hydrogels were prepared and imaged, in order to verify modelling results, and confocal imaging showed comparable distribution of the hydrogels to the expected models. V1 images showed no interspersions of the red fluorescence (acellular hydrogel) and blue fluorescence (cellular hydrogel). As with the simulated results, the hydrogels were distributed in two distinct sections throughout all samples. The cellular hydrogel was situated in one quadrant of the sample while the acellular section resembled an L-shaped bulk (figure 6.5A). V2 images showed that there was a regular interspersed distribution of cellular hydrogel and acellular hydrogel. Samples contained distributions with 3 – 4 cellular hydrogel sections situated around the circumference of the sample, with the acellular hydrogel sections resembling a central cross-shaped bulk (figure 6.5B).

The area percentage for red and blue fluorescence was calculated for 20 quadrants for both groups, and histogram representations of the percentages of blue and red fluorescence present in each quadrant showed that the interspersions of acellular and cellular hydrogel was increased in v2 samples compared with v1 samples (figure 6.5C, D). For v1 samples, quadrants contained either very high or very low percentages of acellular hydrogel area coverage, while for v2 samples there was a greater number of quadrants with medium percentages of acellular hydrogel area coverage. This indicated that there was progression of the design to improve interspersions of the hydrogels to more closely resemble the desired hydrogel sample, and therefore version 2 was selected as more optimal for preparation of the experimental 3° extruded hydrogel scaffolds.

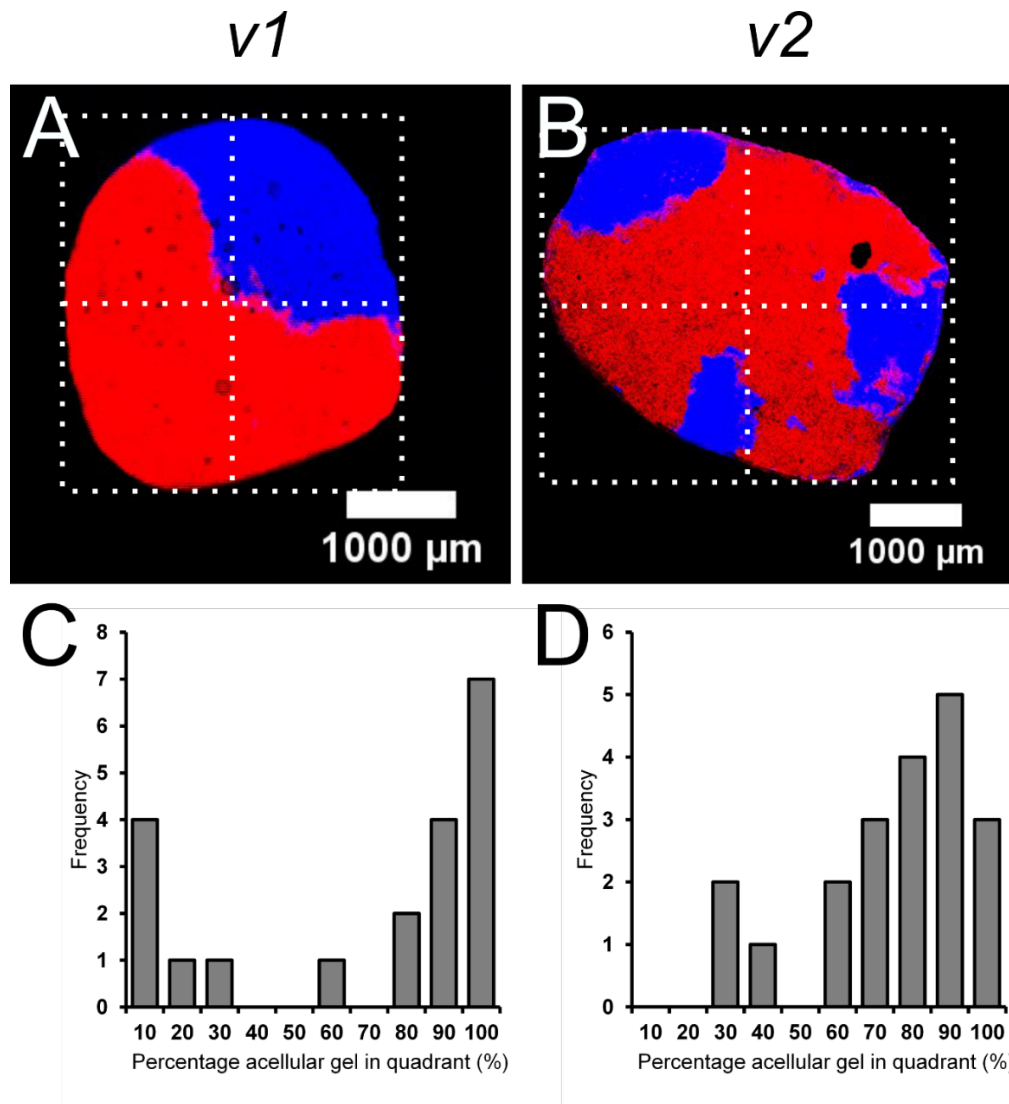


Figure 6.5. Qualitative and quantitative data analysis for the fluorescent bead characterization of the two versions of the 3° extrusion device. Confocal images show the section view, with quadrant selection for *v1* (**A**) and *v2* (**B**) of the 3° extrusion device. Scale bars = 1000 μm . Histograms show the distribution of % acellular hydrogel area coverage in the image quadrants for *v1* (**C**) and *v2* (**D**) confocal image sets. $N = 20$.

Interspersion of the cellular and acellular hydrogel was required for preparation of the 3° extruded hydrogel scaffold. Other authors have attempted to prepare in vitro ordered structures replicating the in vivo nerve regeneration model, using hydrogels as well as other materials. For example, Kitagawa *et al.* used microfluidics and alginate hydrogels of different stiffness to prepare interspersed strips of neuron-like cells [164]. In a similar way, for this investigation, interspersion of the more cell compatible and less cell compatible regions should provide alignment cues for the cell growth in the hydrogel scaffold.

During experimental development, the decision was made to complete 3D printing of both versions of the extrusion device and characterize the system by observing physical extrusion results as well as fluid modelling results. Initially, physical characterization had been selected as the characterization method following prototype design, and subsequently, fluid modelling was added as a secondary method in order to gain a more complete understanding of the flow in the prototypes. Completing 3D printing and physical extrusion characterization alongside fluid modelling took more time and materials than it would have taken if fluid modelling was done before any prototype development; in the latter case, fluid modelling results would have been used to inform selection of one version for 3D printing. However, an advantage of following the former sequence of events is that it provided two characterization methods which could be used to verify and confirm the fluid flow. Considering that there were several approximations made to simplify the *in silico* model, it was useful to have a second dataset collected from independent experiments to give more credence to the characterization and stronger support for the decision of the more suitable device version to complete the cell seeding experiments.

6.5. Cell Distribution and Alignment within the SH-SY5Y Cell Seeded Granular Hydrogels

Resulting neural networks formed within the 3° extruded granular hydrogels (prepared using the modified GG protocol of a 2% (w/v) non-granular gel, granulated using the 40 µm sieve, and combined 1:1 (v/v) with a cell suspension in media and 4% (v/v) PGC, Chapter 5, extruded through version 2 of the 3° extrusion device) were compared with networks formed within non-3° extruded granular hydrogels (prepared using the same modified GG protocol from Chapter 5, but delivered through a syringe). Qualitative fluorescence analysis of distribution of cells in the non-3° extruded setup indicated that the cellular and acellular hydrogel did not occupy distinct locations in the scaffold. Instead, the two hydrogels were mixed and randomly situated throughout the scaffold (figure 6.6A). Furthermore, the distribution of cells within the non-3° extruded hydrogel scaffold was not preferential to areas with cellular hydrogel. Cell nuclei were spread throughout the hydrogel scaffold, and at a lower concentration when compared with the 3° extruded hydrogel scaffold. For the 3° extruded hydrogel scaffold, analysis indicated that the acellular and cellular hydrogels were arranged with areas distinct to either hydrogel group (figure 6.6B). There were more cell nuclei in the cellular hydrogel areas compared with the acellular hydrogel areas, as the scaffold was observed to contain few to no nuclei in the acellular hydrogel granule areas and numerous, densely distributed nuclei in the cellular hydrogel granule areas.

The correlation of cell count with cellular hydrogel granule area coverage (% of total quadrant area) was measured for the two groups, and quantitative results support the visually observed differences in the hydrogels. At both time points, the cell distribution in the 3° extruded hydrogel scaffolds followed a trend: increased cellular hydrogel corresponded with increased cell count. However, for the non-3° extruded hydrogel scaffolds, there was no change of the cell count as the cellular hydrogel concentration increased. Moreover, the cell

counts for the non-3° extruded hydrogel scaffold samples were lower than those for the 3° extruded hydrogel scaffold samples (figure 6.6C, D).

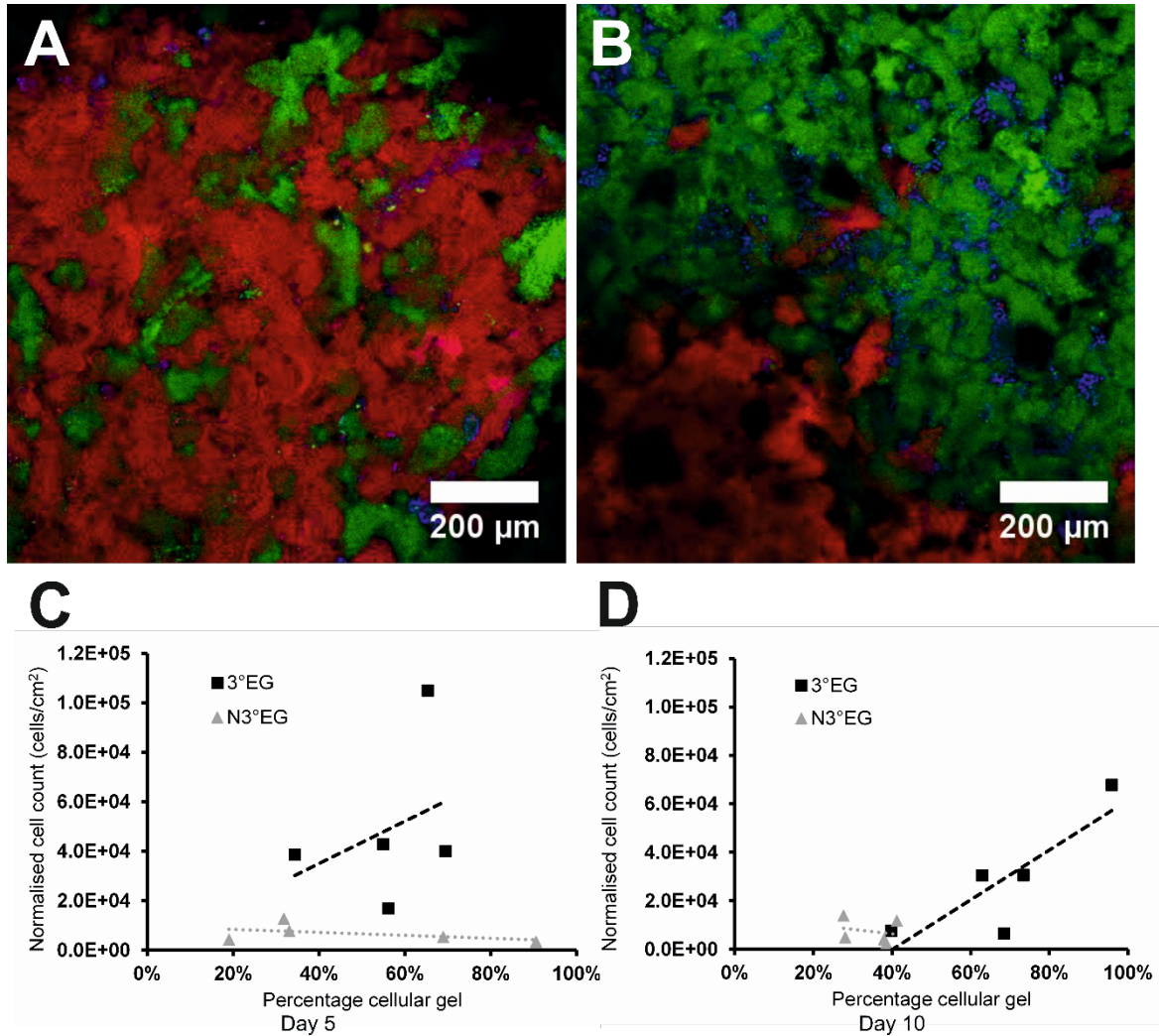


Figure 6.6. Quantification of cell distribution within the hydrogels. Confocal images of the non-3° extruded seeded hydrogel (N3°EG) (A) and the 3° extruded seeded hydrogel (3°EG) (B) taken on d10, shown as summed intensity projections. Green and red colours correspond to green fluorospheres embedded in the cellular hydrogel granules and red fluorospheres embedded in the acellular hydrogel granules. The blue colour corresponds to cell nuclei stained blue with Hoechst. Scale bars = 200 μm. Graphs show XY scatterplots of the cell count against percentage cellular hydrogel on d5 (C) and d10 (D). N = 10.

Immunohistochemistry of the hydrogel scaffolds showed proliferation of the neurons over 14 days of culture. Cells were arranged in groups of two or three cells with neurites extending and connecting with nearby cell groups (figure 6.7A, B). However, visual observation showed higher cell density within the 3° extruded hydrogel scaffold samples compared to cell density within the non-3° extruded hydrogel scaffold samples. Quantitative results confirmed this as the cell density in the non-3° extruded hydrogel scaffolds was significantly lower than density in the 3° extruded hydrogel scaffolds across both time points (figure 6.7C). Moreover, the cell density for the non-3° extruded hydrogel scaffold samples decreased from day 7 to day 14 while the cell density was maintained over the observation period for the 3° extruded hydrogel scaffold samples.

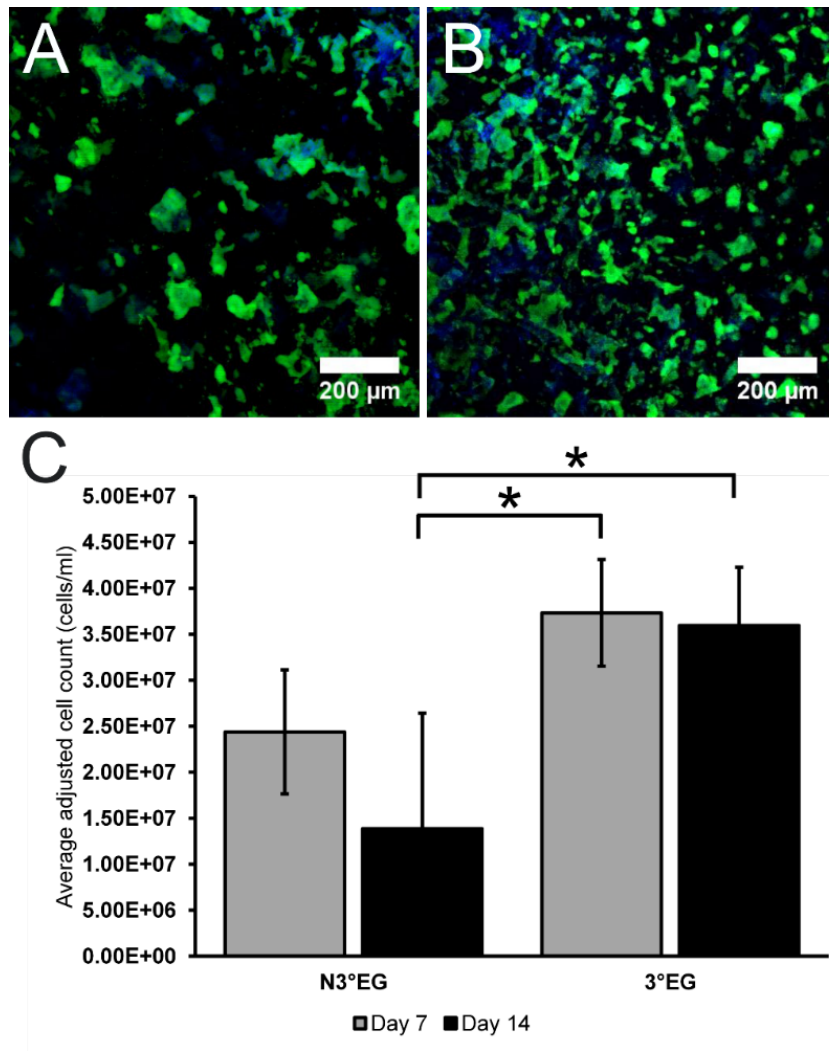


Figure 6.7. Quantification of cell density within the hydrogels. Confocal images show neurons stained green (tubulin) with nuclei stained blue (DAPI), taken after 14 days of culture and shown as summed intensity projections, within the non-3° extruded hydrogel scaffold (N3°EG) (A) and the 3° extruded hydrogel scaffold (3°EG) (B). Scale bars = 200 μm. C. Column chart shows the measured average adjusted cell count (density) for the non-3° extruded and 3° extruded hydrogel scaffold over 14 days of culture. ANOVA Single Factor test showed $p < 0.05$. * Bonferroni corrected post-hoc student's t-tests assuming equal variances showed $p < 0.017$. $N = 20$ cell counts. Error bars represent standard deviation across counts.

The 3° extruded hydrogel scaffold allowed seeding and maintenance of dense cell cultures restricted to the intended areas within the scaffold (within the cellular hydrogel areas). This was possibly due to the physical separation of granules and prepared placement of cells using the 3° extrusion device, as well as the chemotactic differences within the hydrogel granules – the presence of BDNF in the cellular hydrogel granules and the absence within the acellular hydrogel granules.

The physical separation of acellular and cellular hydrogel granules in the 3° extrusion device allowed cells to be embedded between cellular hydrogel granules for some minutes before formation of the full scaffold. Swelling of the hydrogel granules would lock the cells in place, and the start of action of the PGC would further secure the cells, leading to the correlation between cellular hydrogel presence and cell presence (figure 6.6C). Without the separation provided by the 3° extrusion device, as seen in the non-3° extruded hydrogel scaffolds, the cells were not directed to particular hydrogel areas within the full hydrogel scaffold, and therefore were distributed in the mixed cellular and acellular hydrogel areas.

Similarly, the chemotactic environment separation in the 3° extruded hydrogel scaffold bolstered the effect of maintenance of cell distribution within the scaffold compared with the mixing of chemotactic environments in the non-3° extruded hydrogel scaffold. While BDNF was embedded within the cellular hydrogel granules, the neurons would be situated preferentially in these granule areas. In the 3° extruded hydrogel scaffold, the cellular hydrogel areas and therefore the neurons would be separated from the acellular hydrogel areas, resulting in short term maintenance of the cell distribution in the 3° extruded hydrogel scaffold. In the non-3° extruded hydrogel scaffold, the cellular and acellular granules were mixed, resulting in no separation of the BDNF rich areas within the scaffold and no preferential distribution of cells within the scaffold.

The physical and chemotactic structure influencing the cell network structure is likely what allowed longer term maintenance of the cell distribution in the 3° extruded hydrogel scaffold. As the differentiated SH-SY5Y cells proliferated within the hydrogel scaffold over 14 days, the areas with lower cell density would have decreased cell-cell contact and therefore decreased proliferation [173], [174]. This was observed in the non-3° extruded samples, which were observed with even lower cell density at the day 14 time point, implying that the reduced proliferation rate of the cells could not balance the rate of cell death, leading to a reduction in cell density. However, the opposite was seen in the 3° extruded hydrogel scaffolds (figure 6.6D, 6.7C) which maintained the positive correlation between cellular hydrogel presence and cell presence at the day 14 time point. This can possibly be explained by the increased cell density and cell-cell contact resulting in a higher proliferation rate to balance the rate of cell death in these scaffolds.

Alignment analysis of the seeded hydrogels was carried out using the OrientationJ plugin of the ImageJ software to measure coherence of pixels across an angular range -90° to 90°, in collected confocal images modified by maximum intensity projections and auto local thresholding (figure 6.8A, B). Histograms of coherence across angles showed that there was little directional preference in both the N3°E and 3°E hydrogels as pixels were oriented in all directions (figure 6.8C, D). The maximum coherence for hydrogel samples showed low percentages for both hydrogel samples at both time points, further indicating comparably low levels of alignment within the scaffolds (figure 6.8E). The coherence was increased for the 3° extruded hydrogels compared with the non-3° extruded hydrogels for day 7, however this was a non-significant result according to the ANOVA single factor statistical test. On day 14, the maximum coherence value was decreased for the 3°EG compared with the N3°EG.

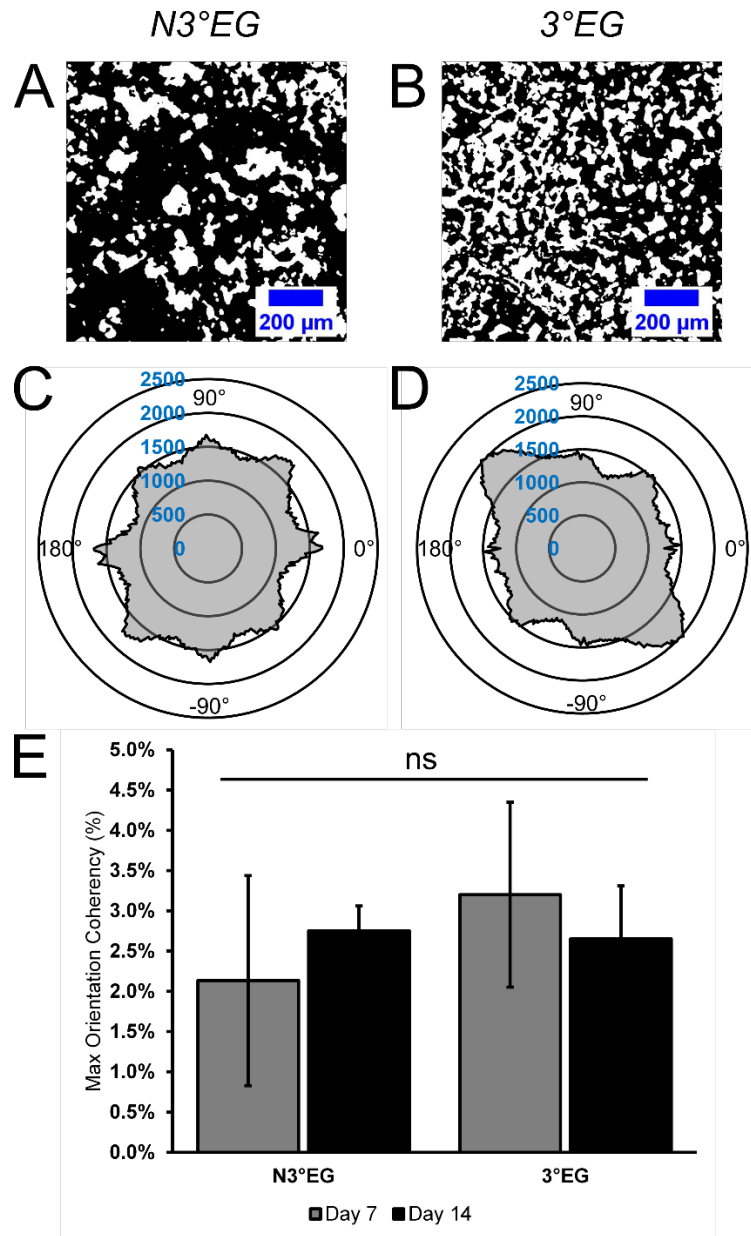


Figure 6.8. Quantification of alignment in the hydrogels. Confocal images post-processing for alignment characterization, shown as maximum intensity auto-local threshold images, for the non-3° extruded hydrogels (N3°EG) (A), and the 3° extruded hydrogels (3°EG) (B). Scale bars = 200 μm . Histograms of weighted coherency, shown as reflected image polar plots for the N3°EG (C) and the 3°EG (D). Concentric rings are labelled with weighted coherency values. E. Column graph showing the average maximum coherency measured for the hydrogels at day 7 and day 14. ANOVA single factor test shows non-significance. $N = 15$ coherency measurements. Error bars represent standard deviation across measurements.

While the 3° extruded hydrogels improved the distribution of cells compared with the non-3° extruded scaffold, the alignment of the cells was not significantly affected by the tertiary structuring of the hydrogel. This was likely because the scale of alignment cue was not strong enough to affect alignment on the single cell scale. Guidance cues within the 3° extruded hydrogel were on the mesoscale – hundreds of microns wide – which can affect the patterning of cell networks but not the patterning of individual cells. However, a combination of mesoscale cues with micro- or nano-scale cues, can work together to direct cell alignment on the single cell scale [175].

6.6. Neural Electrical Activity within the F11 Cell Seeded Hydrogels

F11 cell seeded hydrogel samples were incubated with OGB-1 calcium indicator to record calcium concentration changes within the cells undergoing electrical activity. For this method of observing electrical activity within the neurons, fluorescently marked calcium ions were observed using fluorescent imaging of the cell networks over time, and specific fluorescent fluctuations indicated electrical activity within the cells. Visual investigation of the neural electrical activity in the hydrogel scaffolds showed changes in fluorescence directly related with electrical activity throughout all groups. Cells within each hydrogel sample fired action potentials and were reliably observed over a 180 second time period (figure 6.9A-C, supplementary figures S14-16). Action potentials were selected as portions of the signal with quick exponential rise (< 1 s) from baseline fluorescence values and slower exponential decay (≈ 5 s) and return to baseline fluorescence values (figure 6.9D-F). The majority of spikes were observed as singular spikes expressing the full exponential rise and subsequent return to baseline, however some spikes were observed as combined action potential trains, indicating multiple action potentials occurring in quick succession, the next

action potential beginning before the baseline fluorescence was reached during decay of the previous action potential. Such occurrences were minimal across the groups.

Cells on the TCP control fired action potentials with similar spike rate, not significantly higher than cells within the hydrogels (figure 6.9G, ANOVA, NS), indicating that the hydrogel environment was similar to the control for healthy cell proliferation. However, TCP control cells also fired action potentials with higher spike intensity than cells in the hydrogels, and significantly higher than the cells in the non-3° extruded hydrogels, which might have indicated that the overall activity of the cells within the hydrogels, especially within the non-3° extruded hydrogels, was decreased compared to the cells in the control (figure 6.9H).

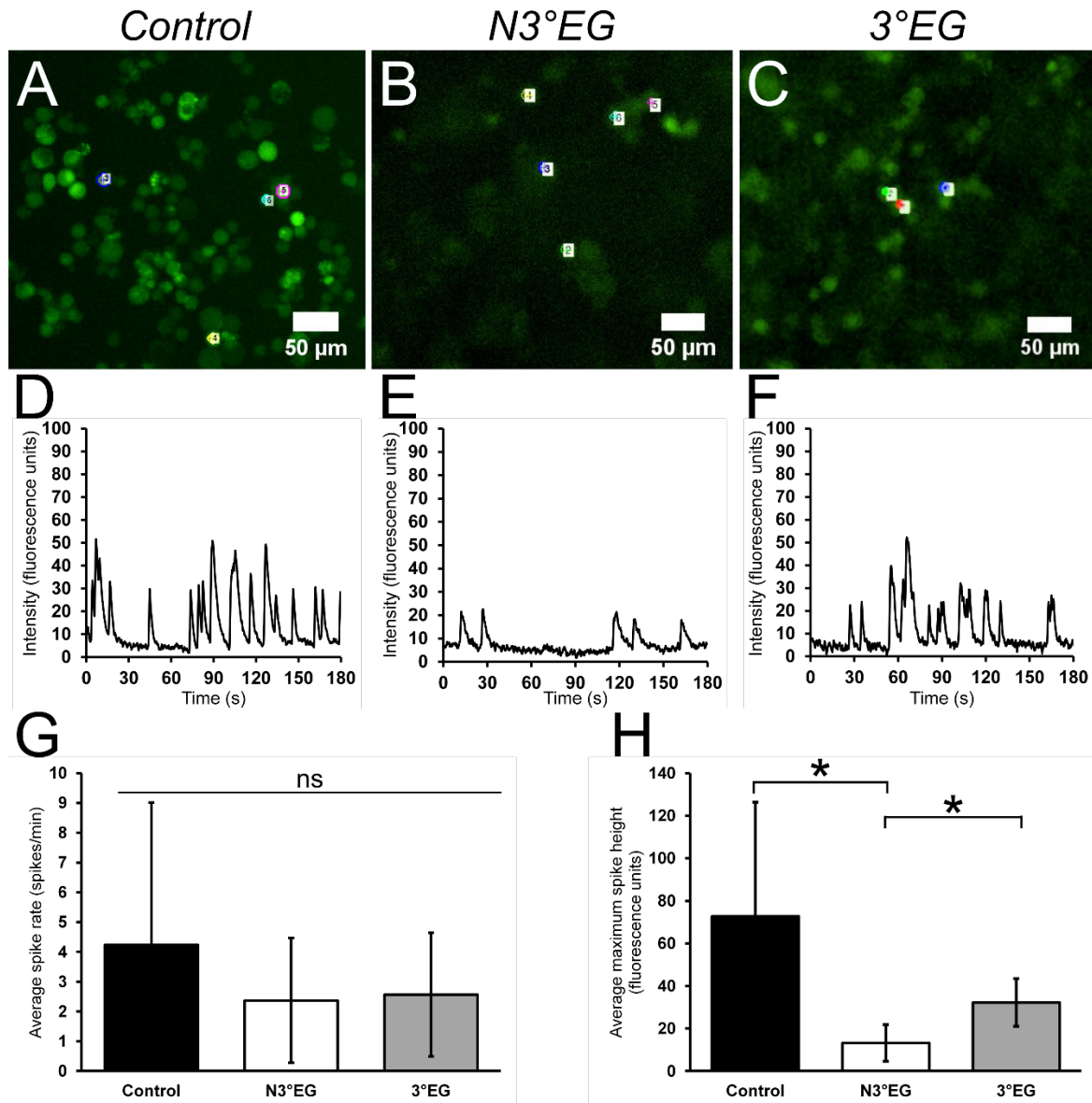


Figure 6.9. Quantification of electrical activity. Screenshot images show selected regions of interest during calcium imaging for the control (A), the non-3° extruded hydrogels (N3°EG) (B), and the 3° extruded hydrogels (3°EG) (C). Green colour corresponds with the cell expression of OGB-1. Scale bars = 50 μm . XY scatterplots show fluorescent activity within the control (D), the N3°EG (E), and the 3°EG (F) over time. Column charts show average spike rate for 10 firing cells in the three investigated groups (ANOVA, NS) (G), and average maximum spike height for the three investigated groups (ANOVA, $p < 0.05$ across groups, *Bonferroni corrected post-hoc student's t -tests, $p < 0.017$) (H). $N = 30$ cell observations. Error bars represent standard deviation across observations.

For the 3° extruded hydrogel samples, this non-significant decrease in spike intensity might be explained by the reduction in fluorescence signal due to higher background signal in the 3d hydrogels [176]. However, it is also likely that the decrease in spike intensity could be attributed to low cell density. This is especially probable for the non-3° extruded hydrogel samples which were observed with even lower cell densities than in the 3° extruded hydrogels (figure 6.7A, C). Given that for increased neural activity, neurons require proximity to other neurons for connected and communicating networks [177], it is understandable that a substantial decrease in cell density would result in a decrease in the neural activity for the non-3° extruded hydrogel samples.

6.7. Assessment of the 3° Extrusion Device and Procedure

Modelling the 3° extrusion device and characterization the hydrogel placement after extrusion showed that the adaptations made for version 2 improved the hydrogel interspersed. The improvements allowed results closely approaching the expectations – production of a hydrogel scaffold with linear cellular hydrogel strips surrounded by acellular hydrogel [164]. For this reason, cell experiments were completed with the v2 of the 3° extrusion device.

The cell experiments showed promising results comparing the 3° extruded hydrogel to the control as well as to the non-3° extruded hydrogel. The 3° extruded granular hydrogel provided an environment that maintained desired distribution of cells within the cellular hydrogel areas over the experimental period (figure 6.6, 6.7) and the hydrogel also maintained cultures that were more comparable to control cultures than the non-3° extruded hydrogel cultures, considering neural electrical activity (figure 6.9). These results indicate that the 3° extrusion device was able to prepare 3° extruded hydrogel samples with organized

structure that better replicates native nerve tissue architecture. These are promising findings that can be applied to improve artificial nerve graft technology.

The tertiary structure of the granular hydrogel was sufficient to control cell distribution, however it was not sufficient to control the alignment of cells (figure 6.8). It has been found that the alignment of cells can be improved by combining multiple directional cues. The mesoscale distribution cues, as experienced by the cells in the 3° extruded hydrogel, can be improved by adding either physical or biochemical microscale cues. For example, Gilchrist *et al.* investigated the combination of multi-scale architectural cues, using a thin hydrogel layer functionalized with selected fibronectin deposition patterns, and found that when the cues were arranged in parallel directions, the combined effect surpassed the effect with only mesoscale cues [175]. Similarly, Rose *et al.* used aligned microgel rods embedded within a hydrogel to improve alignment of fibroblasts within the hydrogel [178]. It can be proposed that implementing microscale alignment cues within the cellular hydrogel would improve the cell alignment capabilities of the hydrogel scaffold.

Furthermore, addition of Schwann cells to the improved 3° extruded scaffold would even more closely replicate the *in vivo* nerve regeneration design. Just as Schwann cells align themselves into bands of Büngner *in vivo*, artificially aligned Schwann cells would provide both physical alignment and chemoattractive cues directing the aligned regrowth of axons entering the hydrogel scaffold. In these ways, the scaffold can be improved to represent an improved artificial nerve graft technology [179].

Overall, the 3° extrusion device produced hydrogel scaffolds with the intended hydrogel mesoscale alignment and desired cell distribution within the hydrogel scaffold. There is potential for improvement of the protocol to achieve cell alignment for the purposes of application to an artificial nerve graft. There are also other applications that can take

advantage of the ability of this device to spatially distribute cell types within a hydrogel. Other tissue models involving cylindrical style co-culture could be created using the 3° extrusion device and granular hydrogel scaffold investigated in this thesis. With minimal modifications to the protocol to include a cell seeded hydrogels in place of the acellular hydrogel, co-culture tissue models for pancreas [180] and vascularized tissues [181], could be designed. Moreover, with further modifications to secondary cylinders in the diffuser plate, other arrangements could be designed such as concentric circles or flat sheets to prepare a plethora of *in vitro* tissue models [20]. This indicates high adaptability of the tertiary extrusion device.

6.9. Chapter Conclusion

This chapter investigated the influence of tertiary structured granular hydrogel scaffold on the cell distribution, alignment, and electrical activity in seeded neural networks. In order to improve organization within the hydrogel and develop a scaffold which more closely replicates peripheral nerve structure, two versions of the extrusion device were prepared and characterized using fluid modelling and fluorescent hydrogel extrusion. Characterization results verified that version 2 prepared a hydrogel scaffold closer to the expectations in terms of hydrogel interspersion. Version 1 resulted in 2 longitudinally adjacent hydrogel masses, while version 2, the extrusion device with addition of a diffuser plate, resulted in 4 cellular hydrogel column interspersed within a cross shaped acellular hydrogel column. Optimization of the device design resulted in a hydrogel scaffold with the desired design characteristics.

Cell seeding in the tertiary extruded hydrogel scaffold and non-tertiary extruded hydrogel scaffold showed that organizing the tertiary structure improved the cell distribution within the scaffold, allowing preferential distribution of the cells within the cellular hydrogel areas of the 3°EG and maintenance of distribution over 14 days. Observation of electrical activity within both scaffolds and a control indicated electrical activity comparable to the control for the 3°EG but less than the control for the N3°EG, potentially caused by low cell density within the sparsely distributed N3°EG hydrogels. Alignment of the cells was not significantly influenced by addition of organized tertiary structure to the hydrogel scaffolds, however there are avenues for improving the scaffold by adding micro- or nano-scale cues to improve cell alignment.

The tertiary extrusion device development and optimization resulted in preparation of a hydrogel with organized structure mimicking regenerating nerve tissue, which formed

distributed and functional neural cultures. These findings indicate that there is potential of this protocol to be used for development of improved nerve graft technology, and the versatility of the extrusion device and granular hydrogel designs also suggest further application to other tissue engineered co-culture models.

Chapter 7: Conclusions and Future Work

7.1. Main Findings and Applications

There is a compelling need to develop relevant *in vitro* tissue models for investigating the human CNS and PNS in order to inform new approaches for restorative treatments. In this thesis, aimed at manipulating primary, secondary, and tertiary scaffold structure for the guidance of neural growth to form complex organized neural networks, two-dimensional and three-dimensional material manipulations were carried out. The resulting neural interaction, neural network formation, cell viability, and cell activity were analysed to determine fulfilment of the aims. Investigations showed that modifications to scaffold structure could direct the growth of neurons to form engineered neural networks.

For the purpose of developing an interface layer based on the machining of micropores into glass coverslips, slope angle was used to test the hypothesis that neurites preferentially explore shallower funnel-shaped micropores. Findings strengthened the postulation that contact guidance of neurites is influenced by growth cone interaction with surface edges [45]. Many investigations concerning neural interactions have focused on microgrooves, aligned fibres, and conical or cylindrical pillars as the topographical surface design [46], [182]–[184]. Few studies investigate neural interaction with micropores, and studies investigating neural interaction with funnel-shaped micropores have not been previously reported [185]. The phenomenon of contact guidance has been used to direct and spatially separate neurites from their cell bodies [130], [182], however, these studies did not investigate the reduction of slope angle to increase the control of neural networks seeded at lower concentrations.

The developed funnel-shaped microporous surface has potential application as a neural interface to a “brain on a bench” model. Due to the ethical issues associated with obtaining live human brain tissue, as well as challenges associated with the use of animal models, the

development of *in vitro* models that have separated interface networks will be of significant benefit. The low-density neural network connected to yet separate from a 3D neural network model, could for instance, function to transfer input signals via light sensitive neurons. While funnel-shaped microporous interfaces are more suitable than pores with steeper angles, it was concluded that fabrication techniques used require further refinement.

For the 3D neural network model layer, granulation was used to improve neurite extension and network formation within the scaffold. To function as a better representative of well-ordered native tissue, tertiary structure was added to the granular hydrogel using extrusion techniques. This structural modification improved cell distribution without detriment to the neural activity within the scaffold. Natural and synthetic hydrogels have been used for cell encapsulation in the non-granular phase, however, using granular hydrogels for cell encapsulation, especially in neural culture, is a more recent development [186]–[189]. Findings demonstrated that a hydrogel with granular structure is more suited than the non-granular hydrogel for improved neurite extension and formation of connected neural networks. The investigated protocol prepares such a hydrogel structure in a quick, repeatable way, with a simpler, top-down fabrication approach, giving it an advantage over current methods.

Based on findings, it was hypothesized that the tertiary extruded granular hydrogel could be applied as a filler material for developed nerve graft tubing, introducing further guidance cues to the inner portion of the nerve graft, and potentially improving the reinnervation of peripheral nerves over longer distances [190], [191]. Furthermore, with the glass micropore surface as a neural interface as previously described, the tertiary extruded granular hydrogel could be applied as a scaffold to culture connected neural networks organized as representative models of parenchymal tissue. Further adjustments to the device design and

cell seeding protocol would make it possible to extend the applicability of the system to a plethora of other *in vitro* models, including co-culture models with vascularized tissues.

7.2. Future Perspectives

There are some challenges remaining that need to be overcome, particularly concerning the laser ablation and HF etching protocol used to prepare samples suitable for full scale development into a functional neural interface. Several iterations of the protocol were made and there was marked improvement. Due to the high variability between intended and resulting funnel widths, as well as challenges associated with fabricating pores that go straight through the coverslip, it is evident that the investigated fabrication protocol needs further optimization. One strategy would be to reconsider the choice of ablation technique. Focused ion beam technology works similarly to femtosecond laser technology, but uses a beam of liquid metal ions instead of photon energy, and offers higher resolution ablation with a trade-off of lengthier ablation time [192]–[194]. The laser ablation technology was initially selected as it was a quicker, less expensive process and it was assumed that the resolution would be sufficient for sample preparation. However, results indicate that an investigation into higher resolution machining technology could improve the accuracy and repeatability of the ablation process, as well as potentially reduce the surface roughness associated with lower resolution machining. Should the improved ablation resolution lead to development of the desired funnel shaped micropores, then an emerging combination laser and ion beam ablation technology could be implemented to decrease the machining time without reducing the resolution [195].

With more time, improvements can also be made concerning the granular hydrogel investigations. A final characterization experiment for the granular hydrogels with different amounts of PGC could be completed in order to gain a more complete view of the change in rheological properties in the hydrogel with protocol modifications and provide further quantitative evidence for the selection of an optimal PGC concentration. Moreover, to enable the application of the tertiary structured granular hydrogel to the development of an improved artificial nerve graft, further design improvements are necessary. Cells were preferentially distributed within the tertiary structured hydrogel scaffold, however, it is evident that further nanoscale alignment cues, such as magnetically aligned rods or glass fibres, are needed to improve the alignment of cells within the distributed hydrogel scaffold [190], [196]. Incorporation of Schwann cells at an optimized concentration should allow the hydrogel scaffold to better replicate the alignment and cellular cues observed *in vivo*. Following this, the optimized hydrogel scaffold could be combined with a silicone or polytetrafluorethylene microporous sleeve or tube, to prepare a prototype for testing [197]. While these investigations were beyond the scope of the thesis given time restraints, the results and techniques developed will be extended further within the research group.

7.3. Summary

This thesis aimed to develop techniques for scaffold preparation that enabled the fabrication of structured neural models. The task was approached from both two-dimensional and three-dimensional points of view to give a global perspective, and investigations tested structural modifications to culture surfaces and scaffolds, increasing their suitability for use in complex cell culture. Findings advance the potential for further collaborative work to develop interfacing technologies using neuronal cells, 3D “brain on a bench” models for

disease modelling and drug-delivery *in vitro* investigation, as well as restorative treatments using structured hydrogels.

Appendices

Supplementary Figures

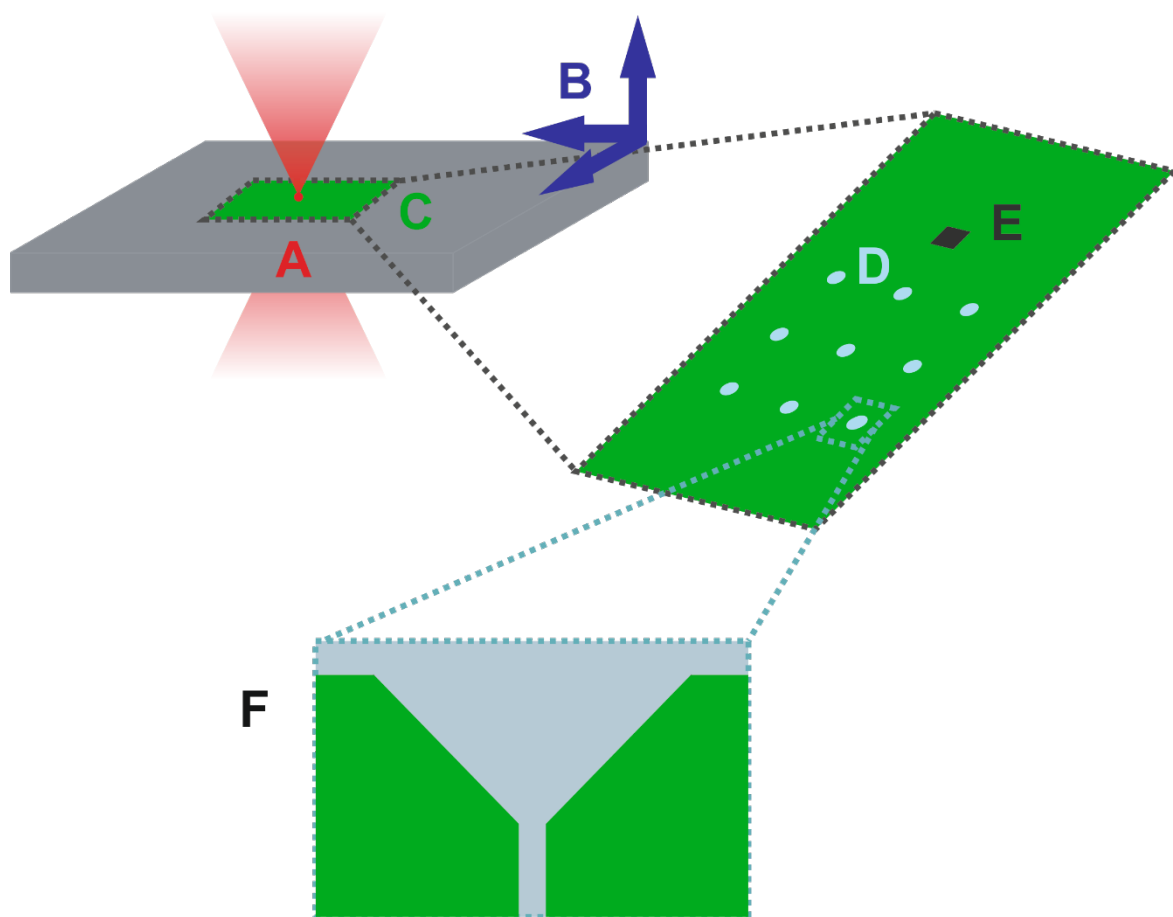


Figure S1. Schematic of the laser machining process. The glass slide is placed in the laser beam focus for ablation of the funnel-shaped pores at set angles. **A.** Laser beam and focus. **B.** Stage movement. **C.** Glass coverslip of 18 mm length and width, 170 μm thickness. **D.** Ablated micropores. **E.** Sample number. **F.** Cross-section of funnel-shaped micropore.

Please note: Figures S2 – S16 are video files attached as supplementary files in the Research Thesis Digital Submission application.

Figure S2-5. Videos showing live imaging of SH-SY5Y cells on 8 μm pore diameter microporous membrane. The cells move across the membrane surface, approaching and moving away from the pore edges. Scale bar = 10 μm .

Figure S6. Video showing live imaging of SH-SY5Y cells on the non-annealed 60 angle group funnel-shaped pores. Cells interact with the pore edge, moving towards, around, and away from the pore edge. Scale bar = 20 μm .

Figure S7. Video showing live imaging of SH-SY5Y cells on the annealed 60 angle group funnel-shaped pores. Cells interact with the pore edge, moving towards, around, and away from the pore edge. Scale bar = 20 μm .

Figure S8. Video showing live imaging of SH-SY5Y cells on the non-annealed 90 angle group funnel-shaped pores. Cells interact with the pore edge, moving towards, around, and away from the pore edge. Scale bar = 20 μm .

Figure S9. Video showing live imaging of SH-SY5Y cells on the annealed 90 angle group funnel-shaped pores. Cells interact with the pore edge, moving towards, around, and away from the pore edge. Scale bar = 20 μm .

Figure S10. Video showing a HA hydrogel granule swelling over time after being placed in PBS. The granule diameter increases, then is maintained. Scale bar = 50 μm .

Figure S11. Video showing live imaging of SH-SY5Y cells seeded on TCP. Cells have extended morphology and form connected networks. Scale bar = 100 μm .

Figure S12. Video showing live imaging of SH-SY5Y cells seeded in NGG. Cells have rounded morphology and do not form well connected networks. Scale bar = 100 μm .

Figure S13. Video showing live imaging of SH-SY5Y cells seeded in GG. Cells have extended morphology and form connected networks. Scale bar = 100 μm .

Figure S14. Video showing fluorescent imaging of F11 cells seeded on the control TCP, incubated with OGB-1. Changes in fluorescence correspond with action potential firing. Scale bar = 100 μm .

Figure S15. Video showing fluorescent imaging of F11 cells seeded on the N3°EG, incubated with OGB-1. Changes in fluorescence correspond with action potential firing. Scale bar = 100 μm .

Figure S16. Video showing fluorescent imaging of F11 cells seeded on the 3E°G, incubated with OGB-1. Changes in fluorescence correspond with action potential firing. Scale bar = 100 μm .

Publications and Presentations

Journal Articles

Julian H George, David Nagel, **Sharlayne Waller**, Eric Hill, H Rhein Parri, Michael D Coleman, Zhanfeng Cui, Hua Ye. A closer look at neuron interaction with track-etched microporous membranes. *Nature Scientific Reports* (2018).

Sharlayne T Waller, Julian H George, Matthew Blundell, Bangshan Sun, Patrick S Salter, Martin J Booth, Hua Ye. Use of laser ablation to generate microporous interfaces for neural applications. (in preparation)

Book Chapter

Julian H. George, **Sharlayne T. Waller**, Hua Ye (2017) 4.13 Use and Manipulation of Microporous Membranes in Mammalian Cell Cultures. In: Drioli, E., Giorno, L., and Fontananova, E. (eds.), *Comprehensive Membrane Science and Engineering*, second edition. vol. 4, pp. 272–292. Oxford: Elsevier.

Conference Presentations

“Using Scanning Electron Microscopy at the Oxford Centre for Tissue Engineering and Bioprocessing”, Oxford LIMA New Users Conference (Oxford, UK, March 2015)

“How to Build a Brain: Engineering microscale features to control neural network patterning and connectivity”, Oxford Interdisciplinary Bioscience Event (Oxford, UK, June 2015)

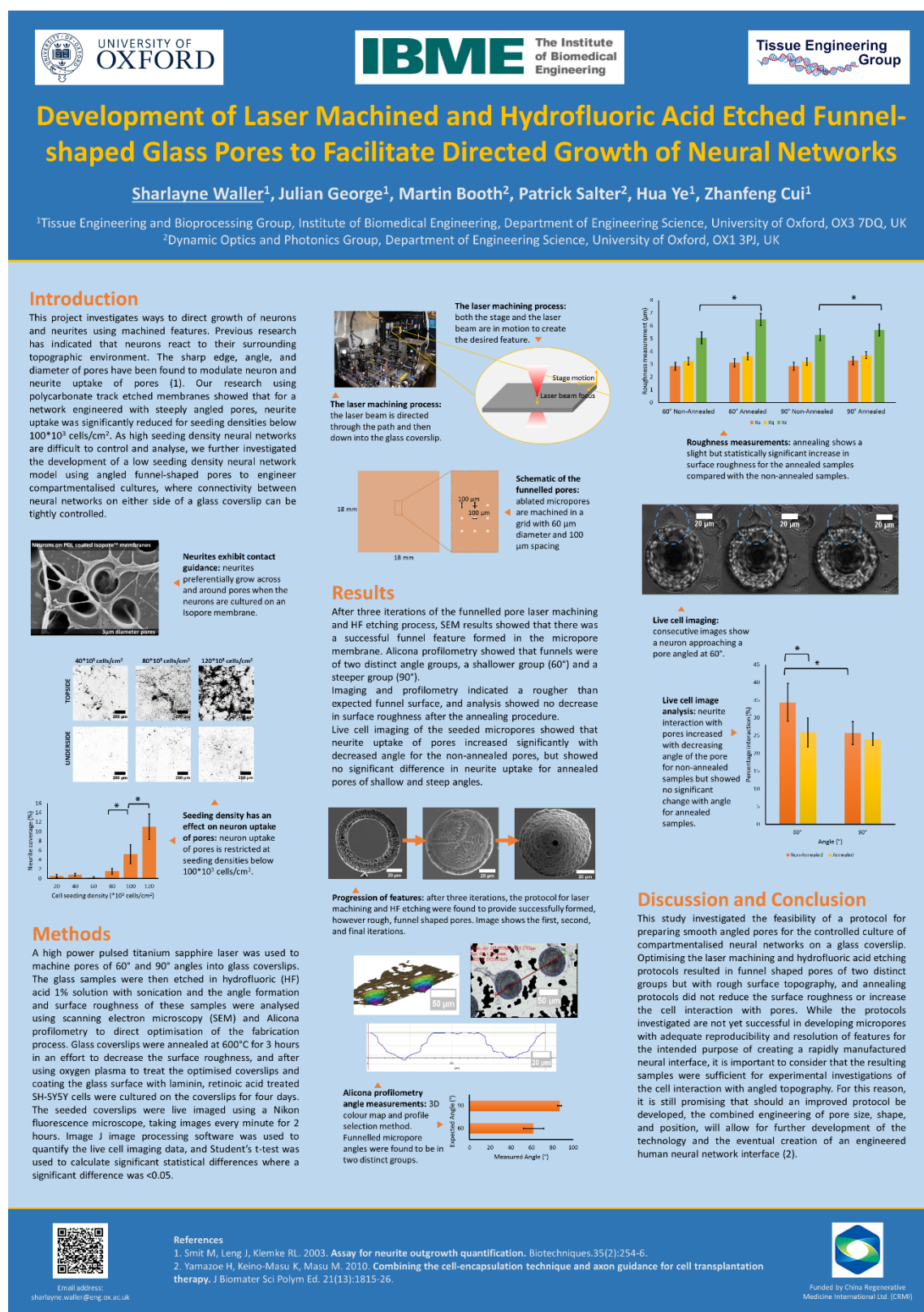


Figure P1. Poster titled “Development of Laser Machined and Hydrofluoric Acid Etched Funnel-shaped Glass Pores to Facilitate Directed Growth of Neural Networks” presented at TERMIS-EU (Davos, Switzerland, June 2017).

Hydrogel granular microstructure used to improve 3D neural network connectivity

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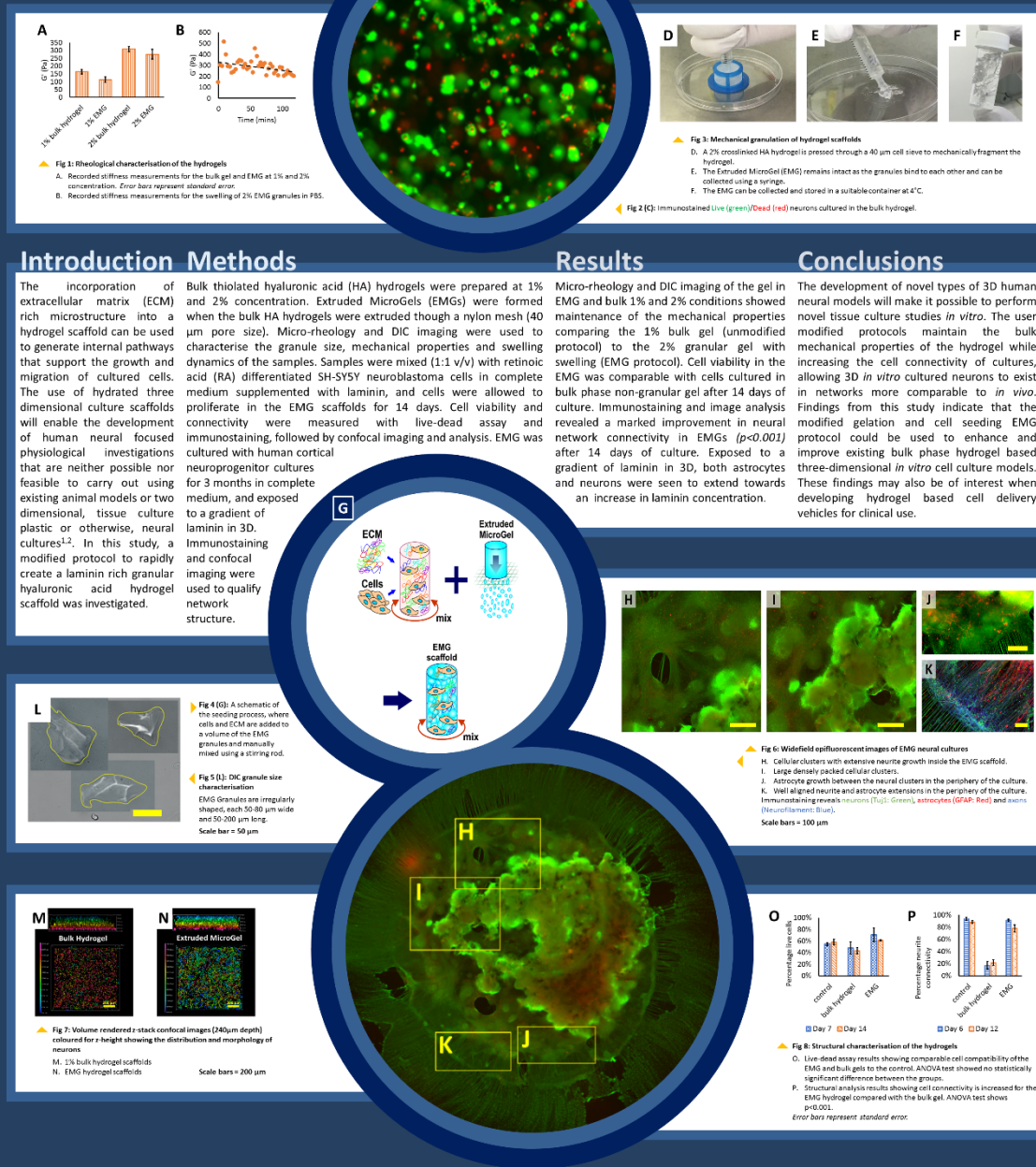


Figure P2. Poster titled “Hydrogel granular microstructure used to improve 3D neural network connectivity”, presented at the 2nd Theodore von Kármán – Discussion Conference on Materials for Life (Bensberg, Germany, April 2018).

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