A 'spinal cord injury in a dish' model to screen neuroregenerative materials

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Abstract

Implantable ‘structural bridges’ based on nanofabricated polymer scaffolds have great promise to aid spinal cord regeneration. Their development (optimal formulations, surface functionalizations, biocompatibility, topographical influences and degradation profiles) is heavily reliant on live animal injury models. These have several disadvantages including invasive surgical procedures, ethical issues, high animal usage, technical complexity and expense. *In vitro* 3-D organotypic slice arrays could offer a novel solution to overcome these challenges, but their utility for nanomaterials testing is undetermined. We have developed an *in vitro* model of spinal cord injury that replicates stereotypical cellular responses to neurological injury *in vivo*, viz. reactive gliosis, microglial infiltration and limited nerve fibre outgrowth. We describe a facile method to safely incorporate aligned, poly-lactic acid nanofiber meshes ($\pm$ poly-lysine + laminin coating) within injury sites using a lightweight construct. Patterns of nanotopography induced outgrowth/alignment of astrocytes and neurons in the ‘dish model’ were strikingly similar to that induced by comparable materials in related studies *in vivo*. This highlights the value of our model in providing biologically-relevant readouts of the regeneration-promoting capacity of synthetic bridges within the complex environment of spinal cord lesions. Our approach can serve as a prototype to develop versatile bio-screening systems to identify materials/combinatorial strategies for regenerative medicine, whilst reducing live animal experimentation.

Keywords: organotypic slice culture, spinal cord injury, *in vitro* model, electrospinning, aligned nanofiber, 3 R’s.

1. Introduction

The implantation of nanofiber scaffolds to serve as synthetic ‘*structural bridges*’ is an approach with high regenerative potential following injury in a range of tissue systems, notably spinal cord injury (SCI) [1]. The latter is a destructive, multifaceted condition, with a poor clinical prognosis for functional recovery [2,3]. Strategically, such scaffolds can aid regeneration by providing aligned topographies, gradients of chemical guidance cues and transplant cell populations to replace lost/damaged cells [4–6]. Evaluation and optimization of synthetic bridges is currently the subject of intensive research globally, with the developmental testing of novel scaffolds and constructs being heavily reliant on live animal injury models [7].

There are several ethical and practical drawbacks relating to animal experimentation in this context. The production of injury models can be a highly invasive and time consuming process, usually requiring high technical expertise. Depending on the model, the procedures can result in serious adverse effects such as infections, paralysis or other movement disorders, bladder dysfunction and so on [7]. Even in the hands of a skilled operator, surgical procedures can inherently generate significant inter-animal variability, requiring large animal group sizes for statistical validity [7]. Following lesion (injury) induction, introduction of synthetic scaffolds into injury areas usually requires re-anesthetization, with the second procedure involving similar risks to injury induction. *In vivo* models necessitate the use of analgesia and rigorous post-operative monitoring of animals, which must be housed individually [8]. The requirements for specialist staff and infrastructure in particular, place major financial constraints on such work [9,10].

Considerations of this nature have prompted the current global drive for the Reduction, Replacement and Refinement of animal experimentation (the 3R’s principles) [11]. In particular, there is a major current need to develop facile, high throughput *in vitro* models

1 that: (i) mimic pathological features of injury sites *in vivo* (and therefore have biological
2 validity); (ii) are compatible with introduction of nano-engineered constructs for the robust
3 and reproducible testing of the latter; and (iii) induce comparable cellular responses to the
4 introduced materials as those in live animal injury models [12].
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10 Models possessing such features can be predicted to reduce animal usage and suffering, as
11 well as costs and technical difficulty, thereby facilitating the screening of pro-regenerative
12 materials for nanomedicine. Despite the need for such biologically relevant testing systems,
13 ‘*reductionist models*’ described to-date typically lack the ability to mimic multifaceted
14 components of SCI pathology and the complexities of cytoarchitecture *in vivo*. The central
15 nervous system (CNS; i.e. the brain and spinal cord) is a particularly challenging tissue
16 system in this regard, due to the complex cellular dynamics and intricate (cardinal)
17 pathophysiological events displayed after neurological injury [13]. For example, following
18 SCI *in vivo*: astrocytes upregulate expression of the astrocyte-specific marker glial fibrillary
19 acidic protein (GFAP), within and adjacent to lesions, to form a scar that constitutes a critical
20 barrier to axonal regeneration [14]; microglia (the immune-competent cells of the CNS)
21 infiltrate into lesion sites and are responsible for the breakdown and phagocytosis of cellular
22 debris and toxic substances following injury [15,16]; and limited, spontaneous sprouting of
23 nerve fibers occurs from lesion margins, with the extent of regeneration declining with age
24 [17].
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46 In this context, 3-D, multicellular organotypic slice cultures (slices of immature tissue that
47 develop comparably to the donor organ in an *ex vivo* environment) could offer a unique
48 solution to the above challenges. For example, such tissue arrays are increasingly being used
49 for long term, high throughput assays in experimental neurology [18]. They provide a
50 versatile bridge between isolated cell culture and *in vivo* experiments wherein the
51 cytoarchitecture and structural relationships of cells are maintained, allowing for parameters
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1 of neural regeneration, e.g. neuronal survival [19], nerve fiber regeneration [20,21] and
2 collateral axon sprouting to be evaluated [22]. These models offer several advantages
3 including the ease of manipulation/observation of *in vitro* preparations [18]; several ages,
4 neuroanatomical areas and species, including human foetuses [23] and transgenic models
5 [24,25] can be used as tissue donor sources, offering high flexibility to study neural
6 pathologies and disease mechanisms. Slice cultures are amenable to electrophysiological
7 techniques [26], molecular biology methods [27], time lapse video microscopy [28] and
8 dynamic confocal imaging [10,29,30], which has greatly expanded the translational utility of
9 this approach. Clearly therefore, such models have wide applicability to a range of tissues and
10 pathologies. Despite their critical advantages, to the best of our knowledge, such models have
11 never been utilized to examine the interactions of nano-materials with cells in an injury-
12 simulated environment.

13 To address this issue, we have established a prototype slice model *in vitro*, which combines
14 for the first time, a tissue injury paradigm with delivery of pro-regenerative scaffolds. To
15 achieve this, we first describe a reproducible method to induce a focal injury in spinal cord
16 slices; the basic pathological features of these injuries *in vitro* have been evaluated to
17 establish their overall relevance to *in vivo* pathology. We then present a novel methodology
18 to incorporate aligned nanofiber scaffolds across injury foci to evaluate the topographical
19 influence on neural cell responses within injury sites.

20 **2. Materials and Methods**

21 **2.1. Materials.** Tissue culture plastics, culture media and supplements were from Sigma-
22 Aldrich (Poole, UK) and Fisher Scientific (Loughborough, UK). Omnipore membranes
23 (JHWP04700) and Millicell culture inserts were from Millipore (Watford, UK). The
24 live/dead cell viability kit was from Invitrogen (Paisley, UK) and Vectashield mounting

medium with DAPI (4', 6-diamidino-2-phenylindole) from Vector Laboratories (Peterborough, UK). Primary antibodies were: rabbit and mouse anti-neuronal class III β -tubulin (clone TUJ-1, Covance, Princeton, NJ), rabbit anti-GFAP (DakoCytomation, Ely, UK), biotin-conjugated anti-lectin (from *Lycopersicon esculentum*, tomato; Sigma-Aldrich, UK). Cy3- and FITC-conjugated AffiniPure secondary antibodies were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA); FITC-conjugated anti-biotin secondary antibody was from Sigma-Aldrich (UK). Poly-L,D-lactic acid (PLA; 96% L: 4% D) was from Purac biochem BV (Gorinchem, Netherlands) and chloroform, dimethylformamide and rhodamine B from Sigma-Aldrich (UK). Collagen type I solution was from BD Biosciences (UK).

2.2. Production of Organotypic Spinal Cord Slice Cultures. The care and use of animals was in accordance with the Animals (Scientific Procedures) Act of 1986 (United Kingdom) with local ethics committee approval. Spinal cords derived from mouse pups aged 0 - 6 postnatal days (P0 - P6) were rapidly removed after decapitation and transferred into ice-cold slicing medium (EBSS buffered with 25mM HEPES) [31–33]. Longitudinal slices (350 μ m) were prepared using a McIlwain tissue chopper. Two/three slices were transferred to Omnipore membrane 'confetti,' resting on the Millicell culture insert membrane. Slices were cultured at the air-medium interface with culture medium (50% MEM, 25% heat-inactivated horse serum, 25% EBSS supplemented with 36 mM D-glucose, 100 U/mL penicillin, 100 μ g/mL streptomycin and 250 ng/mL amphotericin B) for up to 16 days *in vitro* (DIV; Figure 1A1 and A2). Cultures were incubated in humidified 95% O₂/5% CO₂ at 37°C with 80% medium changes every two days. In all cases the number of experiments, *n*, refers to slices obtained across different animals and litters.

2.3. Immunocytochemistry. Slices were washed in phosphate buffered saline (PBS) before and after fixation with 4% paraformaldehyde [PFA; 20 min; room temperature (RT)]. Samples were incubated in blocking solution consisting of 5% normal donkey serum (10% for lectin antibodies) in PBS, with 0.3% Triton X-100 for TUJ-1 and GFAP staining (30 min; RT). Incubations with primary antibodies in blocking solution followed (lectin 1:200, GFAP 1:500; TUJ-1 1:1000; 24 hours at RT or 4°C). Following PBS washes samples were incubated with appropriate Cy3- and/or- FITC-conjugated secondary antibodies (4 h at RT or 4°C). Slices were subsequently washed with PBS and mounted with Vectashield mounting medium containing DAPI.

2.4. Fluorescence Imaging and Statistical Analysis. Slices were visualized on an Axio Scope A1 fluorescence microscope (Carl Zeiss MicroImaging GmbH; Germany) fitted with an Axio Cam ICc1 digital camera and AxioVision software. Where applicable, fluorescence images of immunostained slices were merged using Photoshop CS5.1 (version 12.1). GraphPad Prism v5.0 software was used for all statistical analyses performed. All values quoted are expressed as the mean $\pm$ standard error of the mean (SEM), unless otherwise stated. Data were analyzed by a one-way analysis of variance, using Bonferroni's multiple comparison test for post-hoc analysis; a Bartlett's test was performed to examine homogeneity of variance and, where necessary, data were transformed (square root or log) prior to analysis.

2.5. Assessment of Organotypic Spinal Cord Slice Viability after *In Vitro* Culture. For live/dead assays, intact slices (P0 - P5; n = 3) cultured for 6 – 16 DIV were washed three times with PBS. Slices were incubated with calcein (1 μ L/mL) to label live cells and ethidium bromide (3 μ L/mL) to label dead cells, for 15 minutes at 37°C. Following PBS

1 washes, slices were mounted with Vectashield mounting medium containing DAPI.
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3 Fluorescence micrographs of staining were captured with consistent exposure settings. The
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5 corrected integrated density (Supporting Information S1) was quantified using ImageJ
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7 software (version 1.45s; NIH) and the values for the live-stained micrograph expressed as a
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9 percentage of the sum total from both [30].
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13 **2.6. Lesioning Organotypic Spinal Cord Slice Cultures.** To develop a reproducible focal
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15 lesioning method, a slice lesioning tool was developed in-house using a pre-assembled,
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17 double-bladed scalpel (Figure 1B). This was the product of several prototypes optimized to
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19 rapidly produce regular, evenly spaced lesion margins, whilst preventing drying of slices. The
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21 tool was aseptically assembled prior to lesioning by taping together two surgical blades (size
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23 15) secured into an empty scalpel holder (Figure 1B). To assess the reproducibility of
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25 lesioning tool construction, the diameter of lesions induced in slices was investigated across
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27 five litters (3 - 14 slices per litter; each litter representing independent assembly of the tool).
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29 Fluorescence micrographs of DAPI-stained slices were used to calculate the mean distance
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31 between lesion margins from each culture.
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36 The lesioning procedure was implemented inside a laminar flow hood using a dissection
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38 microscope at x12.5 magnification. To improve lesioning reproducibility, the shape of the
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40 confetti supporting slices (see Figure 1A3 and section 2.2) was re-designed to facilitate the
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42 use of forceps to grip both the culture insert wall and confetti together, thus stabilizing the
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44 slice. The lesioning tool was drawn through the slice and small lateral movements used to
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46 ensure the complete severing of nerve fiber tracts. The slice debris between the two lesion
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48 margins was subsequently removed using an aspirator, fitted with a 200 µl pipette tip.
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51 Cultures were lesioned at 1 - 8 DIV and were fixed within 7 days of lesioning. Slices
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53 generated in spinal cords derived from both 'younger' (P0 mice: lesioned after 1 DIV; fixed 7
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1 days later) and ‘older’ (P5 mice: lesioned after 8 DIV; fixed 7 days later) slices with different
2 extents of intrinsic nerve fiber outgrowth were used for characterizing the interaction of
3 nanofibers with neuronal cells.
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10 **2.7. Quantification of Astrogliosis at Lesion Margins** (Supporting Information S2). P0 - P2
11 slice cultures lesioned after 1 DIV, were fixed at 6 days post-lesioning and stained for GFAP.
12 Slices were imaged with consistent exposure settings and converted to grayscale. Optical
13 density (OD) profiles from the lesion margins were generated using ImageJ software and
14 averaged to form a single profile for each slice. Baseline intensity values were obtained at *ca*
15 1mm from the lesion site, where astrocytes displayed un-reactive morphologies and lower
16 GFAP expression levels. A single, corrected OD profile from the lesion margins of each slice
17 (n = 6) was produced by subtracting the baseline intensity from each value in the averaged
18 trace and averaged with traces from five other slices. The differences in average OD in the
19 zones 0 - 100 μm , 100 - 200 μm and > 200 μm from the lesion boundary were subsequently
20 compared.
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39 **2.8. Quantification of Microglial Infiltration into Lesion Sites.** Spinal cords were extracted
40 from P0 - P2 mice, lesioned after 2 DIV and fixed 0, 5 and 10 days post-lesioning. The
41 numbers of lectin-positive (lectin⁺) microglia were counted within the lesion site of each
42 slice, using a standard size grid overlaid onto each image. The total number of microglia per
43 unit area per slice was averaged at each time point (n = 3).
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53 **2.9. Fabrication and Characterization of Electrospun Nanofibers.** A 2% (w/v) PLA
54 solution was prepared by dissolving the polymer in chloroform prior to the addition of
55 dimethylformamide (7:3 solvent volume ratio). The addition of rhodamine B into the solution
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(0.1 mg/mL) produced fluorescent nanofibers. A parallel electrode collector (Figure 1C1) was used to obtain nanofibers in a highly aligned conformation over a 10 minute unit operation. Electrospinning parameters were kept constant for all experiments. A 'densification' tool (Figure 1C2) was used as an intermediate processing step, to compact the nanofibers from the nanofiber deposition area of the collector (84 cm²) to that of the tool (13 cm²), whilst maintaining the aligned nanofiber conformation. Nanofibers were then mounted onto acetate frames from the densification tool (Figure 1C3) and affixed using a spray adhesive, to be handleable and to maintain their aligned conformation for use in experiments. Nanofibers were desiccated overnight and sterilized in a UV chamber before use in all experiments.

The diameter and line density (number of aligned nanofibers along a distance perpendicular to the axis of nanofiber orientation) of both fluorescent and non-fluorescent nanofibers used were determined from micrographs taken with a field emission scanning electron microscope (Supporting Information S3).

2.10. Nanofiber Surface Treatment with Poly-D-Lysine and Laminin. Multiple sterile, portable nanofiber frames were rapidly incubated with poly-D-lysine and laminin solutions (herein termed PDL and LAM, respectively) on a chamber developed in-house (Figure 1C4). Coating solutions were applied sequentially (20 µg/mL PDL: 12 hours; 10 µg/mL LAM: 5 hours) then washed with PBS and kept moist for placement over slice lesions. Fourier transform infrared spectroscopic analysis confirmed the presence of protein on the surface of treated nanofibers, verifying surface treatment procedures (Supporting Information S4).

2.11. Incorporation of Nanofibers over Lesioned Slices. The technical challenge of incorporating delicate, aligned nanofibers over lesioned slices (Figure 1C5) was overcome by

gently positioning nanofiber-bearing frames parallel to the slice longitudinal axis using forceps. A 3 mg/mL neutralized collagen solution was applied around the acetate frame, to ensure stability for subsequent staining procedures.

2.12. Axonal Outgrowth and Alignment across Lesions ($\pm$ Nanofibers). Spinal cords derived from both ‘younger’ (P0 mice: lesion at 1 DIV) and ‘older’ (P5 mice: lesion at 8 DIV) models were used to evaluate intrinsic nerve fiber outgrowth. Nerve fiber outgrowth density was quantified in TUJ-1 stained slices from both models $\pm$ nanofibers (Supporting Information S5). Fluorescence micrographs were obtained and OD profiles generated from regular intervals along the length of the lesion site using ImageJ software for peak analysis (Supporting Information S5). The average total number of peaks per mm^2 was then calculated for each slice ($n \geq 3$).

To assess the alignment of TUJ-1⁺ nerve fibers with coated and uncoated nanofibers, the percentage of aligned TUJ-1⁺ nerve fibers was scored ($n \geq 3$ per group) by two independent assessors blind to the treatment groups: five data bins, each with a range of 20%, were used to classify any potential alignment observed.

3. Results

3.1. Slice Preparation and Lesion Induction

The viability of intact slices quantified using fluorescence microscopy (Supporting Information S1) was found to be approximately $96\% \pm 2\%$, with a representative slice shown in Figure 2A. Dead cells generated by the slicing procedure were typically found at the slice edges [34].

Following the optimization of the lesioning procedure, a complete transecting lesion could be induced in slices with a double-bladed scalpel, ensuring the severing of nerve fibers across the lesion. In contrast, the use of single-blade cutting instruments, e.g. conventional scalpels, to produce the same injury requires two sequential transecting motions, which increases the procedural difficulty/duration and lesion size variability, whilst restricting the minimum possible lesion size to approximately 750 μm (data not shown). The tissue between the cut edges could be efficiently removed to reveal distinct lesion margins and to enable the visualization of regenerative events across lesion sites (Figure 2B). Measurements of induced lesions revealed a mean distance of 439 μm between lesion margins, which was highly reproducible ($\pm 4 \mu\text{m}$; coefficient of variation = 2 %) across five separate cultures, each representing separate occasions of tool assembly (Figure 2C). Increasing the inter-blade distance concomitantly increased the lesion area, thereby increasing the versatility of the injury model in terms of severity and to accommodate a range of potential scaffold sizes. Live/dead staining of lesioned slices 7 days post-injury (Figure 2D) demonstrated that the procedures did not significantly impact overall slice viability for further experimentation, as evidenced by a central band of live cells with few dead cells interspersed around the area of injury.

3.2. Characterization of Slice Lesion Pathology

Following the development of the lesioning procedure, a series of neuropathological assessments were performed to evaluate whether the following key pathological events known to occur *in vivo* were mimicked within slice lesions.

(i) *Increased astrocyte reactivity in lesion margins*: Clear and even GFAP staining was observed throughout slices; notably, GFAP⁺ cells at lesion margins were intensively reactive

and hypertrophic (Figure 3A) - hallmark features of the glial scar *in vivo* [35]. Quantification of relative GFAP expression at slice lesion margins versus normal areas in the body of the slice (Supporting Information S2), using mean fluorescence intensity profiles (Figure 3B; n = 6) revealed a significant intensity increase in the first 100 μm adjacent to lesion margins (Figure 3C). Comparatively, in regions more distant to the lesion site, GFAP⁺ astrocytes with normal, polygonal morphologies and lower fluorescence intensities were found.

(ii) *Microglial activation and infiltration into lesion sites:* Lectin positive (lectin⁺) microglia were identified in immunostained slices and displayed activated morphologies within the lesion site (Figure 3A). In contrast, resting microglia located in the main body of slices exhibited numerous ramified processes. Counts of the numbers of lectin⁺ microglia within the lesion sites of slices (n = 3 per time point) revealed a significant increase in the number of microglia at 5 days post-lesioning (Figure 3D), with a decrease in number after 10 days.

(iii) *Age/time-dependent spectrum of intrinsic nerve fiber outgrowth from lesion margins:* After seven days of culture, nerve fiber outgrowth in young slices was extensive and randomly orientated (Figure 3E). By contrast the outgrowth in older slices was relatively limited, but also with random orientations (Figure 3F).

3.3. Incorporation of Nanofiber Scaffolds over Lesions and Topographical Influences on Cells

The introduction of rhodamine B into the PLA solution to fabricate fluorescent nanofibers had no statistically significant (two-tailed Student's t-test) effect on either diameter (566 ± 20 nm *versus* 534 ± 5 nm for non-fluorescent nanofibers) or line density (534 ± 5 fibers / mm

versus 566 ± 20 fibers / mm for non-fluorescent nanofibers) of nanofibers produced (Supporting Information S3), highlighting reproducibility in nanofiber production/processing for all experiments.

Nanofibers were mounted onto acetate frames (Figure 4A), with both the fluorescence and alignment (Figure 4B) retained post-culture with lesioned slices (Figure 4C). The overall viability after incorporation of uncoated nanofiber scaffolds over lesioned slices (Figure 4D) remained high, with comparatively few dead cells present around the lesion site and within the body of the slice. Some evidence of cellular attachment to nanofibers across the lesion was also demonstrated (Figure 4D; white arrow heads), indicating nanofiber-slice contact over the culture period.

Evaluation of the topographical influence of the nanofabricated scaffolds on cells in lesion sites (using uncoated versus PDL-LAM coated fibers; $n \geq 3$ slices in each treatment group) showed that gliotic scar formation occurred similar to control slices (without nanofibers), with an intense region of GFAP expression at the first 100 μm of lesion margins. Following incorporation of uncoated nanofibers, no evidence of astrocyte attachment/alignment was observed (Figure 4E). In striking contrast, PDL-LAM coating of nanofibers induced extensive alignment of astrocytes, notably extending long thin processes (c.a. 100 - 200 μm) across the lesion site (Figure 4F). Extensive attachment of microglia to both uncoated (Figure 4G) and coated nanofibers was observed over lesion sites. Notably, elongated microglia were visible over the entire area of the slice in contact with nanofibers.

Quantification of the outgrowth (Supporting Information S5) and alignment of nerve fibers in both models of intrinsic regeneration showed that no significant nerve fiber attachment and outgrowth occurred (Figure 5A) following incorporation of uncoated nanofibers. In striking contrast, coated nanofibers enhanced nerve fiber outgrowth (Figure 5B), as confirmed by the mean nerve fiber outgrowth density (Figure 5C). Semi- quantitative assessment of the

alignment of nerve fibers on coated/uncoated nanofibers (Figure 5D) showed extensive alignment in both lesion models on coated nanofibers: topographical effects appeared more pronounced in the lesion model generated from P0 mice compared to that generated from P5 mice, where a greater incidence of interaction between nerve fibers and materials were observed. Additionally, a sub-population of aligned cells displayed the morphological phenotypes of spinal cord interneurons (Figure 5E). Occasionally, evidence of nerve fiber extension coincident with elongated astrocytes was observed (Figure 5F), suggesting that a component of nerve fiber elongation may occur secondary to topographical cues from aligned astrocytes.

4. Discussion

'Combinatorial' neural tissue engineering strategies have been suggested to be essential to promote various aspects of neural regeneration (such as nerve fiber regeneration, suppression of scar formation and immune responses, promotion of blood vessel growth) within the complex, multi-faceted pathology of SCI [36–40]. Such synergistic approaches have the potential to regenerate the injured spinal cord with varying degrees of efficacy, but none have been successfully translated into the clinic [41]. The full potential of combinatorial strategies utilising aligned nanofibers with combinations of cells and biomolecules has yet to be elucidated, due in part to a heavy reliance on *in vivo* SCI models, in the absence of high-throughput, biologically-relevant *in vitro* screening models of SCI [5,42]. Two-dimensional reductionist tools in current widespread use e.g. microfluidic devices, have provided useful insights in tissue engineering, as these permit the study of fundamental, isolated aspects of neuronal regeneration and response to materials/biomolecules post-injury [43,44]. However, such *in vitro* models lack simulation of more complex multicellular pathology, within a

1 relevant extracellular injury environment, for detailed readouts of the biological response to
2 materials.
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4 By contrast, we consider that the SCI model developed here can be exploited to address
5 this important technological gap. First, 5-7 spinal cord slices can be routinely obtained from a
6 single animal (depending on age), permitting the assessment of several conditions within the
7 same batch of slices, thereby reducing experimental variability and successfully addressing
8 the 3R's principles. Second, we demonstrate that three cardinal pathological features of SCI
9 *in vivo* can be mimicked in slice lesions, viz. post-traumatic astrogliosis, infiltration of lesions
10 by activated microglia (which is broadly comparable with their acute infiltration
11 characteristics *in vivo*) [33,48] and limited random outgrowth of nerve fibers from the lesion
12 margins of slices derived from older animals.
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26 Third, the cellular responses observed in slice lesions following incorporation of PLA
27 scaffolds or laminin-coated scaffolds are comparable to published reports *in vivo*, using a
28 complete transecting injury model (a model used widely in experimental neurology to
29 evaluate regenerative strategies). PLA is approved for clinical use by the Food and Drug
30 Administration as it creates non-toxic waste products and has been widely used as a
31 scaffolding material in the tissue engineering research community. Uncoated PLA scaffolds
32 generally exhibit low levels of host neuronal regeneration and typically result in the
33 formation of a gliotic scar at the interface between host tissue and the implant [46–49]. By
34 contrast, the incorporation of laminin (or a suitable hydrogel e.g. fibrin [50]) into bridges
35 increases axon regeneration and disrupts gliotic scar formation *in vivo* [51–54]. PDL has
36 additional effects in promoting neuronal cell adhesion [55]. The decision to use both coatings
37 in this study was based on reports in the literature that suggest the attachment and extension
38 of neuronal processes is enhanced on PDL-LAM coated surfaces, compared to PDL alone
39 [56]. The extensive attachment of microglia to nanofibers bears resemblance to the activity of
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microglia *in vivo* following transplantation of nanofiber scaffolds into SCI sites [57]. The observation that cells in the 3-D slices can distinguish between different surface coatings suggests that they are able to make sophisticated choices regarding material interactions, within a complex environment *in vitro*. This highlights the high utility of our model in acting as a reliable '*predictor*' of *in vivo* neural cell behaviours in response to various materials and surface chemistries, and hence its value as a bio-screening method. Furthermore, this suggests that the model can enable comparative investigations of various modifications to enhance nerve fiber outgrowth and alignment, including: different polymer formulations and other potentially efficacious substrates with an aligned topography; fiber densities and diameters; scaffold functionalization with therapeutic biomolecules (promoting growth or targeting major CNS inhibitors) and therapeutic stem/progenitor cell populations.

The combination of the portable and lightweight nanofiber meshes with supporting acetate frames utilized in this study provide an innovative solution for determining the functional utility of various nanofiber materials. The post-collection processing of aligned nanofibers to-date has been heavily reliant on direct collection onto 2-D glass coverslips for mechanical support, or alternatively, relatively thick, 3-D, more mechanically stable nanofiber meshes have been utilized but have additional challenges regarding cellular infiltration throughout the mesh thickness [1,58,59]. The supporting acetate frames: (i) provide mechanical stability to the nanofibers; (ii) maintain nanofiber alignment throughout culture; (iii) obviate the requirement for including a supporting substrate (e.g. a gel) to bridge the lesion gap, which would add to the complexity of the construct and increase the difficulty of interpreting the basic pro-regenerative readout of different nanofiber materials and surface treatments on multiple neural populations across injury sites; and (iv) permit a choice of nanofiber density. The density of nanofibers chosen in this study was fine tuned in order to: (i) provide sufficient nanofiber surfaces for cells to interact with without obscuring the visualization of

cellular events in lesions and; (ii) reduce nanofiber clumping and hence retain the topographical influence on neural cell populations. The development of a coating chamber for these studies permits the efficient and sequential coating of multiple nanofiber frames in suspension with different biomolecules.

In terms of the utility of the two models of different ages chosen for our study, the extensive random nerve fiber outgrowth in younger slices is suited to the examination of axonal outgrowth on bioengineered substrates with an aligned topography. By contrast, the older slices that recapitulate cardinal neural features of traumatic injury in the adult CNS, such as limited nerve fiber outgrowth, are suited to the examination of the regeneration-enhancing properties of novel biomaterials. The spontaneous sprouting of nerve fibers observed from the margins of lesioned spinal cord slices in this paradigm, which declines with the age of donor tissue and is influenced by pre-lesioning culture time, has been reported previously [31]. Further, functional assessments of regeneration i.e. electrophysiological recordings may provide a more detailed readout of scaffold regeneration-enhancing properties within this model [26]. Both models are suitable for studying the responses of the non-neuronal, (supporting) glial cells in lesions. The nanofiber-induced morphological reorganization of scar-forming astrocytes that normally form a critical barrier to nerve fiber regeneration reveals a potential application of the injury model for screening efficacious molecules and strategies that aim to disrupt the neuroglial scar, via the re-organization of reactive astrocyte morphology. Further, the observed infiltration of microglial cells into lesion sites indicates that acute inflammatory responses can be mimicked within the lesion sites *in vitro*. Their activation in SCI can be a significant barrier to the development of efficacious interventions as there is evidence implicating them as inhibitors of axonal regeneration via expression of inhibitory guidance molecules such as Netrin-1 and repulsive guidance molecules [60,61]. Microglial attachment to nanofibers provides an *in vitro* readout

1 for the optimization and testing of biocompatible materials and coatings that evoke minimal
2 inflammatory responses. It also demonstrates the potential for long-term studies, where
3 preliminary examination of the material degradation properties and breakdown mechanisms
4 may be assessed.
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9 Whilst this study has utilized spinal cord as the test tissue, we consider that the high-
10 throughput model described here can serve as a prototype for the wider development of
11 highly versatile bio-screening systems for regenerative medicine/nanotechnology. We can
12 predict that these will allow for the identification of novel pro-regenerative materials for a
13 wide range of tissue applications, whilst significantly reducing reliance on live animal
14 experimentation, thereby accelerating the rate of discovery of nanotherapeutic agents for
15 tissue engineering (Figure 6).
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30 **5. Conclusion**

31 We have developed a multicellular, *in vitro* model of spinal cord injury that mimics
32 multiple cardinal features of *in vivo* pathology. Functionalized nanofibers were able to induce
33 dramatic responses in multiple cell types in the injury sites; these are comparable to those
34 induced in live animal models. Our studies demonstrate the high potential of the model to
35 function as a prototype screening system for promising nanotherapeutic interventions, either
36 in isolation, or as part of a combinatorial treatment strategy. We can predict that the use of
37 higher-throughput *in vitro* models of SCI, such as the one we describe here, can aid in
38 overcoming a growing bottleneck in the therapeutic testing of promising new materials and
39 combinatorial therapies, whilst reducing the high current reliance on live animal testing.
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60 **Acknowledgments**

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Figure 1. The production and lesioning of organotypic spinal cord slice cultures. (A) Schematic diagram depicting: (1) the slicing of murine spinal cords into 350 μm sections in the longitudinal sagittal plane and; (2) their transfer to culture inserts. (3) Following a defined culture period 'x', slices were lesioned with a double bladed scalpel by holding an elongated section (red arrow) of confetti (purple) together with the wall of the insert (grey) using forceps, thus keeping the slice stationary. (B) Photograph of the two scalpel blades, demonstrating equal blade spacing along the length of the blades. (C) Schematic diagram depicting: (1) electrospinning of aligned fluorescent poly-L,D-lactic acid nanofibers onto a parallel electrode collector; (2) the use of a densification tool (blue) to increase the line density of collected nanofibers; (3) attachment of portable acetate frames (black) containing spray adhesive to the aligned nanofibers on the densification tool (blue); (4) polymer coating of individual nanofiber frames in a specially designed chamber; (5) placement of aligned nanofiber frames over lesioned slices.

Figure 2. Characterization of spinal cord slices and induction of focal lesions. (A) Representative fluorescence micrograph of a live/dead-stained slice at 6 DIV, showing predominantly live cells in the main body of the slice with dead cells typically found at the slice edges (white arrowheads). (B) A slice stained with DAPI at 2 days post-lesioning, showing clear demarcation of lesion margins (white broken lines). (C) Bar chart of the distances between lesion margins, showing the reproducibility of the lesioning procedure across different experiments, each involving different slice preparations and freshly assembled lesioning tools ($n = 5$). (D) Representative live/dead stained fluorescence micrograph of a slice 5 days post-lesioning reveals some dead cells in lesion sites (white broken lines) with high viability in the main body of the slice.

Figure 3. Characterization of the cardinal features of SCI pathology in lesioned slices. (A) Representative fluorescence micrograph of a lesion margin 12 days post-lesioning shows intensely reactive astrocytes expressing an increase in GFAP (astrocyte marker; white arrowheads) expression and displaying hypertrophic morphologies (red arrowheads). The concomitant infiltration of rounded, lectin (microglial marker) expressing, activated microglia into the lesion site can be seen (white arrows). (B) Line graph of the optical density profiles for GFAP⁺ astrocytes in lesioned slices 7 days post-lesioning (mean profile in red; n = 6) showing a peak in expression at lesion margins. (C) Bar graph showing a significant difference between the average optical densities for GFAP⁺ slices between the first 100 μ m from the lesion margins and two adjacent regions further into the slice body (**p < 0.001; n = 6). (D) Bar graph quantifying numbers of lectin⁺ microglia in lesion sites at 0, 5 and 10 days post-lesioning, demonstrating a peak in infiltration at 5 days (*p < 0.05, **p < 0.01; n = 3 per time point). (E) Representative fluorescence micrograph of extensive, random outgrowth of TUJ-1⁺ (pan-neuronal marker) nerve fibers in lesions in young slices (P0; lesioned after 1 DIV; stained 7 days later). (F) Representative fluorescence micrograph of relatively limited, random outgrowth of TUJ-1⁺ nerve fibers in older slices (P5; lesioned after 8 DIV; stained 7 days later).

Figure 4. Assessment of the cellular responses in lesions after placement of nanofibers over injured slices. (A) Photograph of aligned fluorescent nanofibers adhered to portable acetate frames. (B) Representative fluorescence micrograph of aligned nanofibers adhered to portable acetate frames. (C) Fluorescence micrograph showing placement of portable, aligned, uncoated nanofibers over a lesioned slice. (D) Live/dead staining of a lesioned slice 3 days after placement of aligned uncoated nanofibers, verifying safe nanofiber-placement procedures (white arrowheads mark cells likely to be in contact with nanofibers across the lesion). (E)

Representative fluorescence micrograph of GFAP⁺ scar-forming astrocytes shows no interaction with aligned uncoated nanofibers. (F) Representative fluorescence micrograph showing the attachment, outgrowth and alignment of scar-forming GFAP⁺ astrocytes on poly-D-lysine/laminin coated nanofibers across slice lesions. (G) Representative fluorescence micrograph showing attachment and alignment of lectin⁺ microglia to uncoated nanofibers.

Figure 5. The effect of nanofiber coating on the outgrowth and alignment of TUJ-1⁺ nerve fibers. (A) Representative fluorescence micrographs of the same field showing limited attachment and alignment of TUJ-1⁺ nerve fibers (left-hand panel) to uncoated aligned nanofibers (right-hand panel) in an ‘older’ lesion model displaying limited intrinsic regeneration (P5 slices; lesioning and nanofiber placement after 8 DIV; fixed 7 days later). (B) Representative fluorescence micrographs of the same field showing extensive outgrowth and alignment of TUJ-1⁺ nerve fibers (left-hand panel) on aligned coated nanofibers (right-hand panel) in the same lesion model as in (A). (C) Bar chart quantifying TUJ-1⁺ nerve fiber outgrowth density across lesions with un-coated and coated nanofiber (NF) treatment groups, compared to controls without nanofibers, in both ‘younger’ (P0 slices; lesioned after 1 DIV) and ‘older’ (P5 slices; lesioned after 8 DIV) slice models (**p < 0.01, ***p < 0.001). (D) Bar graph showing the distributions in scores of TUJ-1⁺ nerve fiber alignment for both un-coated and coated nanofiber treatment groups in both younger and older models. (E) Representative fluorescent micrograph of a likely TUJ-1⁺ interneuron at the lesion margins of control slices (P5) without nanofibers. (F) Fluorescence micrograph showing incidences of TUJ-1⁺ nerve fiber contact-guidance with aligned GFAP⁺ astrocytes (white arrowheads) in lesions with coated nanofibers.

Figure 6. Schematic diagram illustrating the potential screening utility of a ‘neural injury-nanomaterial’ interface paradigm.

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