Alignment of Multiple Glial Cell Populations in 3D Nanofiber Scaffolds: Towards Development of Multicellular Implantable Scaffolds for Repair of Neural Injury

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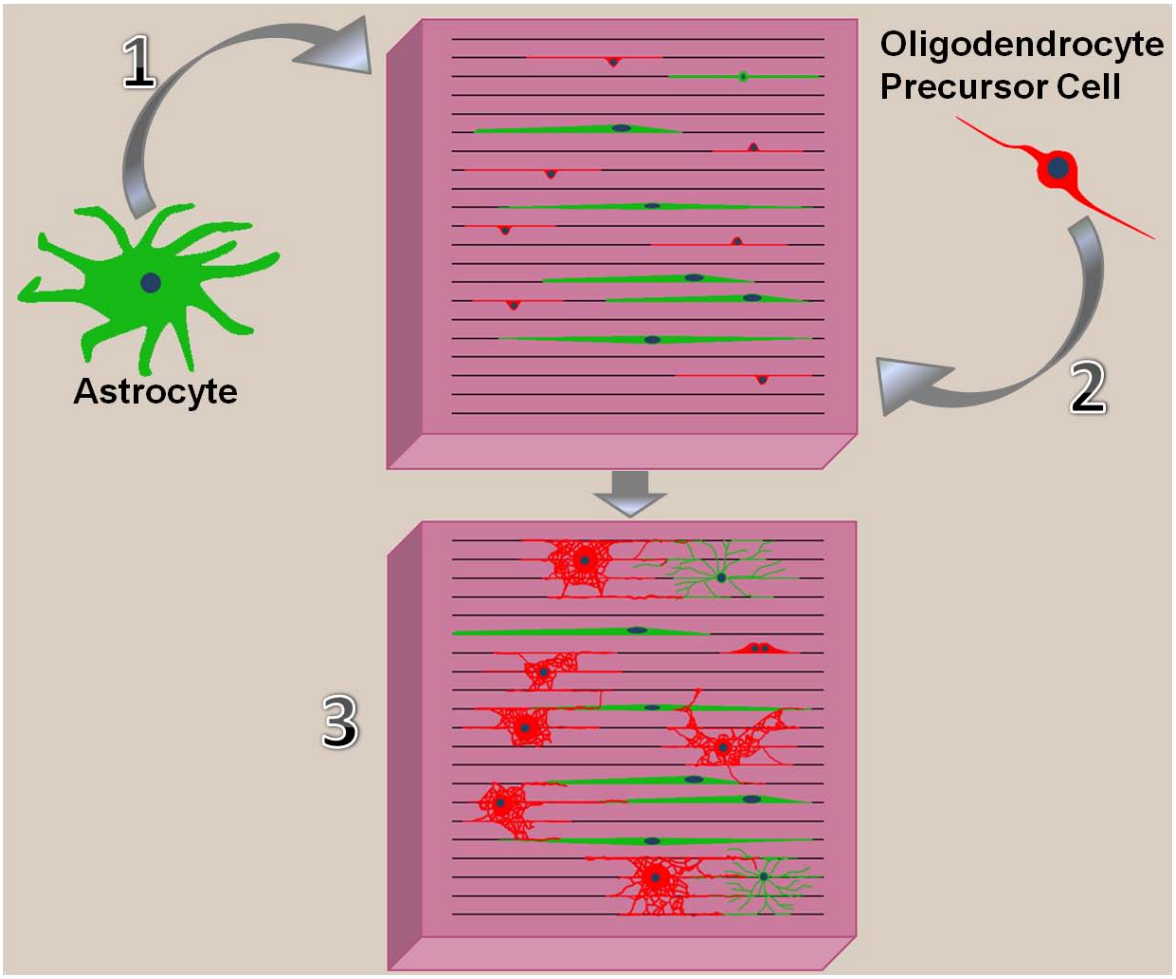
Graphical Abstract



Schematic diagram depicting the production and culture of complex 3D multi-cellular constructs using a sequential cell seeding procedure. Astrocytes (1) were seeded on aligned nanofiber-collagen hydrogel constructs followed by addition of oligodendrocyte precursor cells (OPCs; (2)) after astrocyte alignment. The multicellularity of the constructs containing OPCs, mature oligodendrocytes, type I and type II astrocytes is shown in (3).

Abstract

Non-neuronal cells of the central nervous system (CNS), termed 'neuroglia', play critical roles in neural regeneration therefore replacement of glial populations via implantable nanofabricated devices (providing a growth-permissive niche) is a promising strategy to enhance repair. Most constructs developed to date have lacked three-dimensionality, multiple glial populations and control over spatial orientations, limiting their ability to mimic *in vivo* neurocytoarchitecture. We describe a facile technique to incorporate multiple glial cell populations [astrocytes, oligodendrocyte precursor cells (OPCs) and oligodendrocytes] within a three-dimensional (3D) nanofabricated construct. Highly aligned nanofibers could induce elongation of astrocytes, whilst OPC survival, elongation and maturation required pre-aligned astrocytes. The potential to scale-up the numbers of constituent nanofiber layers is demonstrated with astrocytes. Such complex implantable constructs with multiple glial sub-populations in defined 3D orientations could represent an effective approach to reconstruct glial circuitry in neural injury sites.

Key words: Neuroglia, nanofiber scaffolds, electrospinning, neural regeneration, 3D implant

Background

The prognosis for recovery from spinal cord injury (SCI) is often poor due to the cord's limited intrinsic regenerative capacity.[1] Following acute SCI, a cascade of pathological events can occur that damage the local microenvironment and the supporting non-neuronal cells of the central nervous system (CNS), collectively termed 'neuroglia', which play vital roles in regeneration following injury (e.g. the clearance of cellular debris, genesis of the insulating myelin sheath around regenerating nerve fibers and synaptogenesis).[2,3] Therefore, a promising strategy to enhance regeneration is to reconstruct glial cell circuitry in lesion sites via the implantation of organized glial cell constructs. Currently, a major strategy to achieve this goal in neural tissue engineering studies utilizes highly aligned electrospun nanofibers for the attachment/alignment of individual glial cell populations, e.g. astrocytes.[4]

Despite the therapeutic potential of this approach, investigations of glial alignment on polymer scaffolds have overwhelmingly utilized 2D substrates.[5] Additionally, there are few reports of scaffolds functionalized with multiple populations of aligned CNS neuroglia, to reconstruct the glial circuitry. This study describes a new technique to co-culture astrocytes, oligodendrocyte precursor cells (OPCs) and oligodendrocytes, derived from primary cultures, within 3D collagen-hydrogel scaffolds incorporating portable aligned nanofiber meshes that can spatially organize the cells by contact guidance for reconstruction of glial circuitry. Further, the potential for scaling-up constructs with multiple stacked nanofiber layers is demonstrated with aligned astrocytes.

Materials and methods

Both fluorescent and non-fluorescent, aligned poly-L,D-lactic acid nanofibers were produced by electrospinning and transferred onto acetate frames at two different densities (see Supplementary Material section S1 for detailed description). Primary mixed glial cultures were generated for the purification of astrocytes or OPCs[6] (see S2); the care and use of all animals was according to the Animals (Scientific Procedures) Act of 1986 (UK) with local ethics committee approval. All data were derived from three independent cultures, each established from a different rat litter.

Single layer nanofiber-collagen hydrogel (nanofiber-hydrogel) scaffolds seeded with astrocytes and/or OPCs were fabricated to assess the aspect ratios[5] of aligned astrocytes and OPCs, or their co-cultures, compared to control hydrogel scaffolds without nanofibers, after immunocytochemical staining (S3-S6). Briefly, the nanofiber-hydrogel scaffolds were produced by placing a single acetate

frame with affixed nanofibers on top of a 3 mg/mL collagen type I hydrogel base (S3), with cells subsequently seeded onto each scaffold and cultured in D-10 medium. For co-culture experiments, astrocytes were pre-seeded for two days before the addition of OPCs. Constructs were processed at two or eight days after OPC addition.

A schematic diagram of the multiple nanofiber-layered construct assembly containing astrocytes is shown in Figure 1A (methods: S4). Briefly, each nanofiber layer (Figure 1B) was stacked perpendicular to the adjacent layer (Figure 1C) with a square filter-paper frame in between each layer. Collagen solution was added to the center of the construct to stabilize the nanofibers (Figure 1D). A live/dead cell viability kit and confocal microscopy were used to observe astrocyte segregation and orientation over the three layers, after two weeks of culture.

Results

Both fluorescent and non-fluorescent nanofibers (ϕ250-500 nm)[7] were obtained in a highly aligned conformation using the parallel electrode collector setup and adhered to portable, handleable acetate frames (S1). The fabrication of layered 3D constructs was feasible without disrupting the nanofiber alignment. The nanofibers remained aligned throughout culture and fixation, with the fluorescence detectable during post-culture analysis (Supplementary Figure 1 and Supplementary video 1).

Astrocytes showed association with, and attachment to nanofibers approximately four hours post-seeding. Both glial fibrillary acidic protein-positive ($GFAP^+$) type I and II astrocytes could be observed in the cultures. Significant cellular alignment with evidence of cell proliferation on nanofibers was observed over the following four days (Figure 2A & inset). Astrocytes seeded onto control hydrogel constructs generally maintained rounded morphologies over the observation period (Figure 2B & C).

The aspect ratios of astrocytes grown on nanofiber-hydrogel constructs exhibited higher median values with a relatively wide distribution compared to those on control hydrogels (Figure 2C). The lengths of aligned astrocytes were significantly higher on nanofiber-hydrogel constructs compared to controls (Figure 2D).

Strikingly, OPC mono-cultures lacked evidence of elongation at all time points in both control and nanofiber-hydrogel constructs (data not shown). Despite extensive attachment to nanofibers by four days (> 80%), the majority of cells appeared rounded and phase dark, with extensive cell clumping

and debris, suggestive of cell death (data not shown). Further, the majority of OPCs seeded on control hydrogel constructs containing pre-seeded astrocytes displayed rounded morphologies and lacked bipolarity (Figure 3A). By contrast, the addition of OPCs to nanofiber-hydrogel constructs containing astrocytes pre-aligned for two days dramatically promoted OPC survival, attachment and elongation over the subsequent two days of co-culture. Cells with the distinctive bipolar morphology of OPCs with evidence of cell proliferation on nanofibers could be clearly observed from 24 hours (Figure 3B & inset). An analysis of the aspect ratios of individual OPCs co-cultured with astrocytes shows morphological profiles similar to aligned astrocytes, although with aspect ratios and lengths being smaller in magnitude (Figure 3C & D).

When the period of astrocyte-OPC co-culture was extended to eight days, OPCs displayed the potential to differentiate into complex process-bearing Myelin Basic Protein positive (MBP^+) oligodendrocytes elaborating large sheets of membrane. Such cells appeared to contact both nanofibers and pre-aligned astrocytes (Figure 3E). Astrocytes with distinct type I and II morphologies could also be found (Figure 3E inset).

Astrocytes were successfully seeded sequentially onto multiple, stacked nanofiber layers. Clear spatial separation of the individual nanofiber layers in perpendicular arrangements and associated astrocyte alignment could be detected in all three layers (Figure 4; Supplementary videos 1 and 2).

Discussion

This study outlines a facile and convenient technique to generate a 3D nanofabricated construct, utilizing portable, highly aligned nanofiber layers to control the spatial orientation of multiple glial cell populations. Here we provide ‘proof-of-principle’ that co-cultured primary astrocytes and cells of the oligodendroglial lineage can all survive, proliferate and elongate on the designed constructs, representing an enhancement in spatial and cellular complexity compared with other nanofiber-based scaffolds reported to-date.[8]

The survival/elongation of OPCs seeded as a single population on both nanofiber-hydrogel constructs and control hydrogels was limited, and the reasons for this are not clear. However, these cells showed significant survival and alignment following co-culture with aligned astrocytes, an observation consistent with the major supporting roles played by astrocytes *in vivo*[9] and in mixed glial cultures as the supporting bed layer,[6] suggesting aligned astrocytes may provide survival cues for OPCs. Over an

extended culture period, the maturation of OPCs into complex membrane elaborating phenotypes was observed, suggesting intricate intercellular cross-talk that can support the survival and development of neuroglial populations within the constructs. Additionally, in preliminary experiments we have found that it is feasible to further enhance the cellular complexity of the constructs with seeded primary microglial cells that showed significant attachment and survival following co-culture with the above glial populations (data not shown). Therefore, we consider that the constructs developed here have a significant capacity to support the growth and development of a range of neural cell types. A growing body of literature demonstrates that elongated populations of astrocytes significantly enhance neuronal outgrowth.[5] The enhancement effect of aligned Schwann cells on peripheral neuronal cell migration and myelination has also been observed.[10] Thus, inclusion of oligodendrocyte lineage cells in aligned nanofibrous scaffolds holds promise for enhancing neural repair through the neuroprotective effects exerted by oligodendrocytes and their potential to enhance functional recovery through myelination of regenerating nerve fibers.

In summary, the present construct offers an enhanced order of glial complexity by providing an aligned topography, which has the potential to bridge and guide axonal outgrowth across lesions through a potentially repair-mediating environment consisting of multiple elongated, glial cell populations. Further work is required to refine the multiple-nanofiber layered constructs with a range of glial sub-types and evaluate their effects to enhance neuronal growth and myelin sheath formation in neurological injury models.

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References

1. Yiu G, He Z. Glial inhibition of CNS axon regeneration. *Nat Rev Neurosci* 2006;**7**:617-27

2. Shibuya S, Yamamoto T, Itano T. Glial and axonal regeneration following spinal cord injury. *Cell Adh Migr* 2009;**3**: 99-106

3. Pfrieger FW. Roles of glial cells in synapse development. *Cell Mol Life Sci* 2009;**66**:2037-47

4. Gerardo-Nava J, Führmann T, Klinkhammer K, Seiler N, Mey J, Klee D, et al. Human neural cell interactions with orientated electrospun nanofibers *in vitro*. *Nanomedicine (Lond.)* 2009;**4**:11-30

5. East E, de Oliveira DB, Golding JP, Phillips JB. Alignment of astrocytes increases neuronal growth in three-dimensional collagen gels and is maintained following plastic compression to form a spinal cord repair conduit. *Tissue Eng Part A* 2010;**16**:3173-84

6. McCarthy KD, de Vellis J. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* 1980;**85**:890-902

7. Yang Y, Wimpenny I, Ahearne M. Portable nanofiber meshes dictate cell orientation throughout three-dimensional hydrogels. *Nanomedicine: NBM* 2011;**7**:131-36

8. Kubinová S, Syková E. Biomaterials combined with cell therapy for treatment of spinal cord injury. *Regen Med* 2012:**7**;207-24

9. Wang DD, Bordey A. The astrocyte odyssey. *Prog Neurobiol* 2008;**86**:342-67

10. Daud MF, Pawar KC, Claeyssens F, Ryan AJ, Haycock JW. An aligned 3D neuronal-glial co-culture model for peripheral nerve studies. *Biomaterials.* 2012;33(25):5901-13

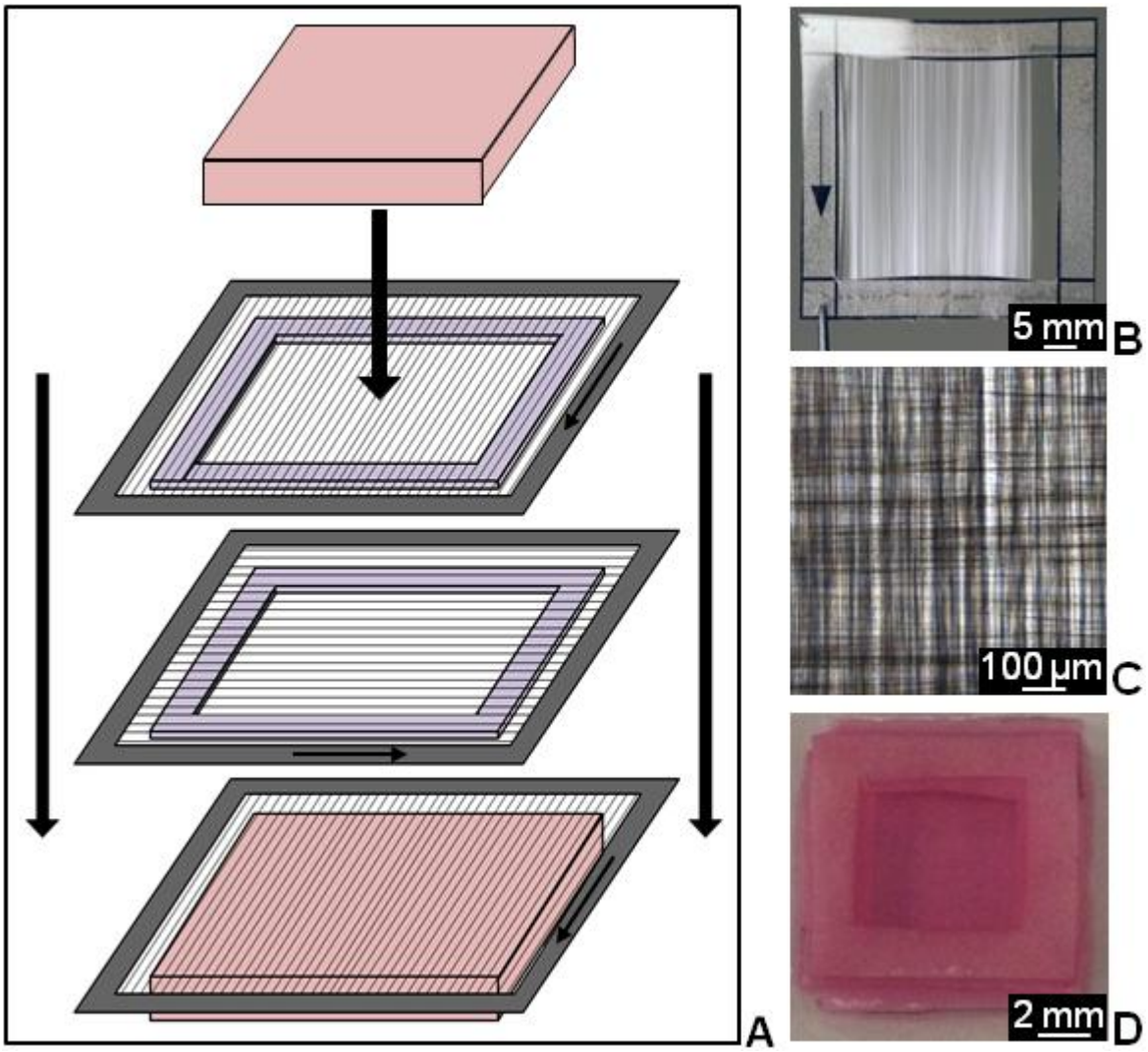
Figure Legends

Figure 1: 3D nanofiber-hydrogel constructs using stacked layers of nanofiber meshes to align cell populations. (A) Schematic diagram depicting the assembly of three nanofiber layer constructs. Cells were seeded sequentially onto each nanofiber mesh (dark grey) with spacers in between layers (light purple) and collagen (pink) added last to the center of the construct. (B) 16 cm^2 acetate frame with a mesh of adhered, highly aligned nanofibers. (C) Phase micrograph of perpendicularly stacked nanofibers. (D) Photograph of the assembled three nanofiber layer construct.

Figure 2: Astrocyte elongation in nanofiber-hydrogel constructs. (A) Fluorescence micrographs of the proliferation (inset) and elongation of astrocytes over the four day culture period. (B) Fluorescence micrograph of $GFAP^+$ astrocytes on control hydrogel constructs after four days of culture. (C) Individual astrocyte aspect ratios on control and nanofiber-hydrogel constructs. (D) The mean ± standard error (SEM) of the lengths of astrocytes on control and nanofiber-hydrogel constructs (***$p < 0.001$).

Figure 3: OPC elongation and maturation following culture with pre-seeded astrocytes on nanofiber-hydrogel constructs. (A) Fluorescence micrograph of control hydrogel constructs pre-seeded with $GFAP^+$ astrocytes for two days, followed by two days of co-culture with $A2B5^+$ OPCs. (B) Fluorescence micrographs of OPCs and pre-seeded astrocytes on nanofiber-hydrogel constructs cultured for the same time as controls (inset showing OPC proliferation). Note the bipolar phenotype of the precursor cells. (C) Individual OPC aspect ratios on nanofiber-hydrogel constructs containing pre-aligned astrocytes. (D) The mean (± SEM) lengths of OPCs in nanofiber-hydrogel constructs. (E) Fluorescence micrographs after eight days of co-culture, showing the co-existence of astrocytes and oligodendrocytes on nanofiber-hydrogel constructs with presence of complex, highly processed, membrane-elaborating phenotypes, characteristic of mature oligodendrocytes (for comparison, see precursor forms in Figure 3B). White arrows indicate potential contacts between aligned oligodendrocytes and astrocytes. (Inset) Clear morphological distinction between type II astrocytes (green arrowhead; left), type I astrocytes (white arrow; center), and oligodendrocytes (red diamond arrow; right) within the same microscopic field.

Figure 4: Fluorescence micrograph merged over a series of z-stack confocal images, showing live/dead stained astrocytes after two weeks of culture. Cellular alignment with three distinct (stacked) nanofiber layers orientated perpendicular to each other is observed.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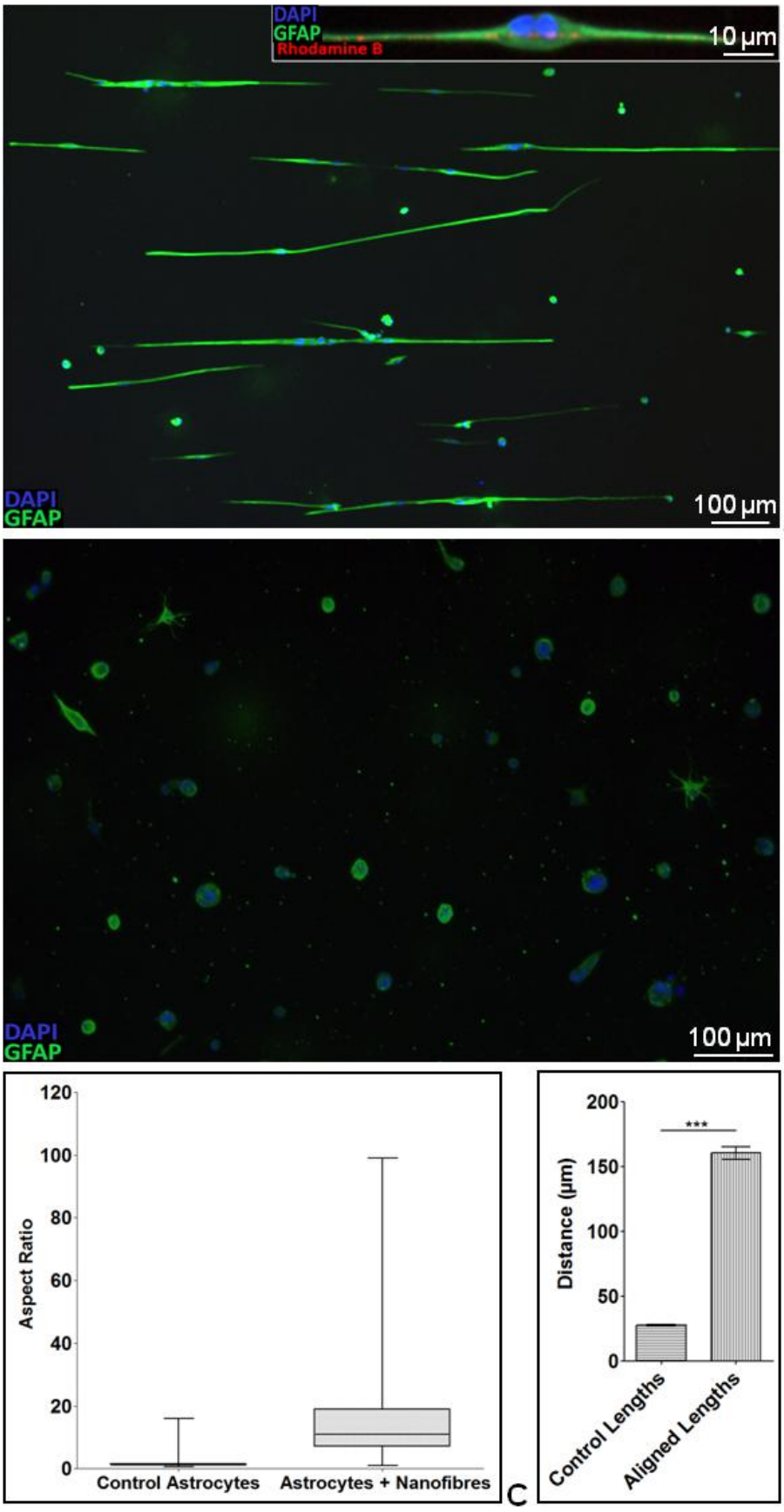
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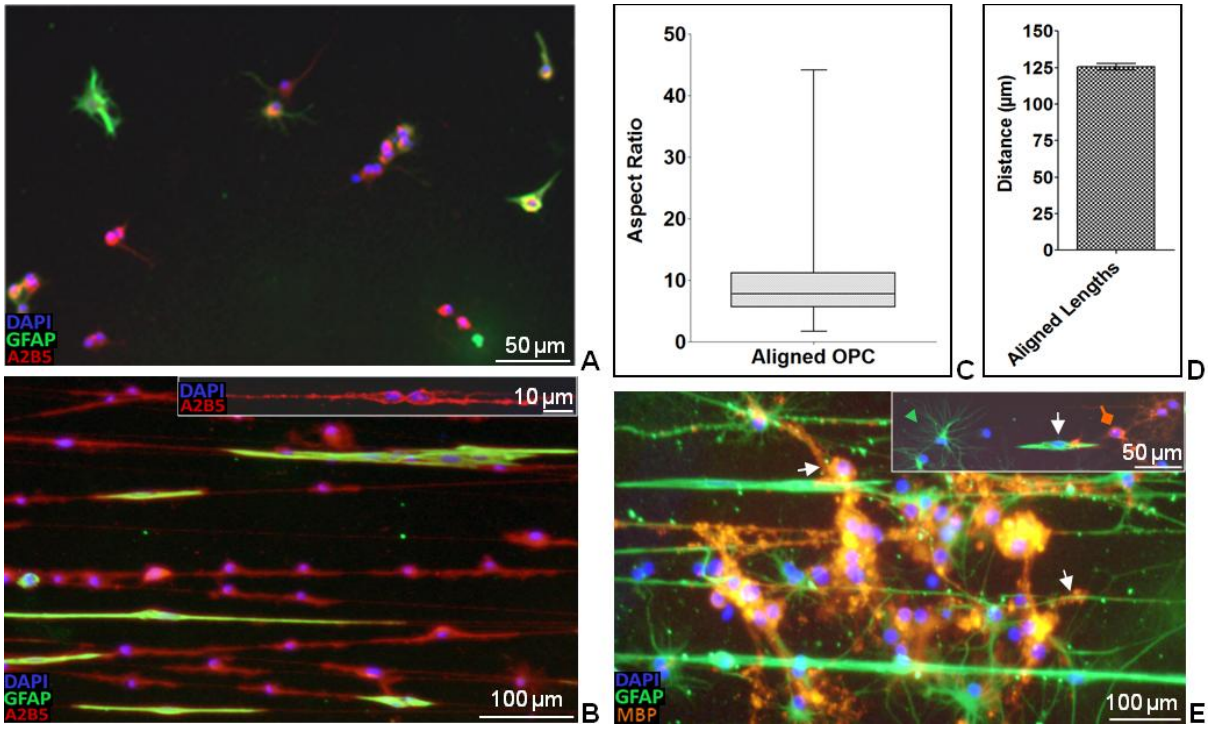
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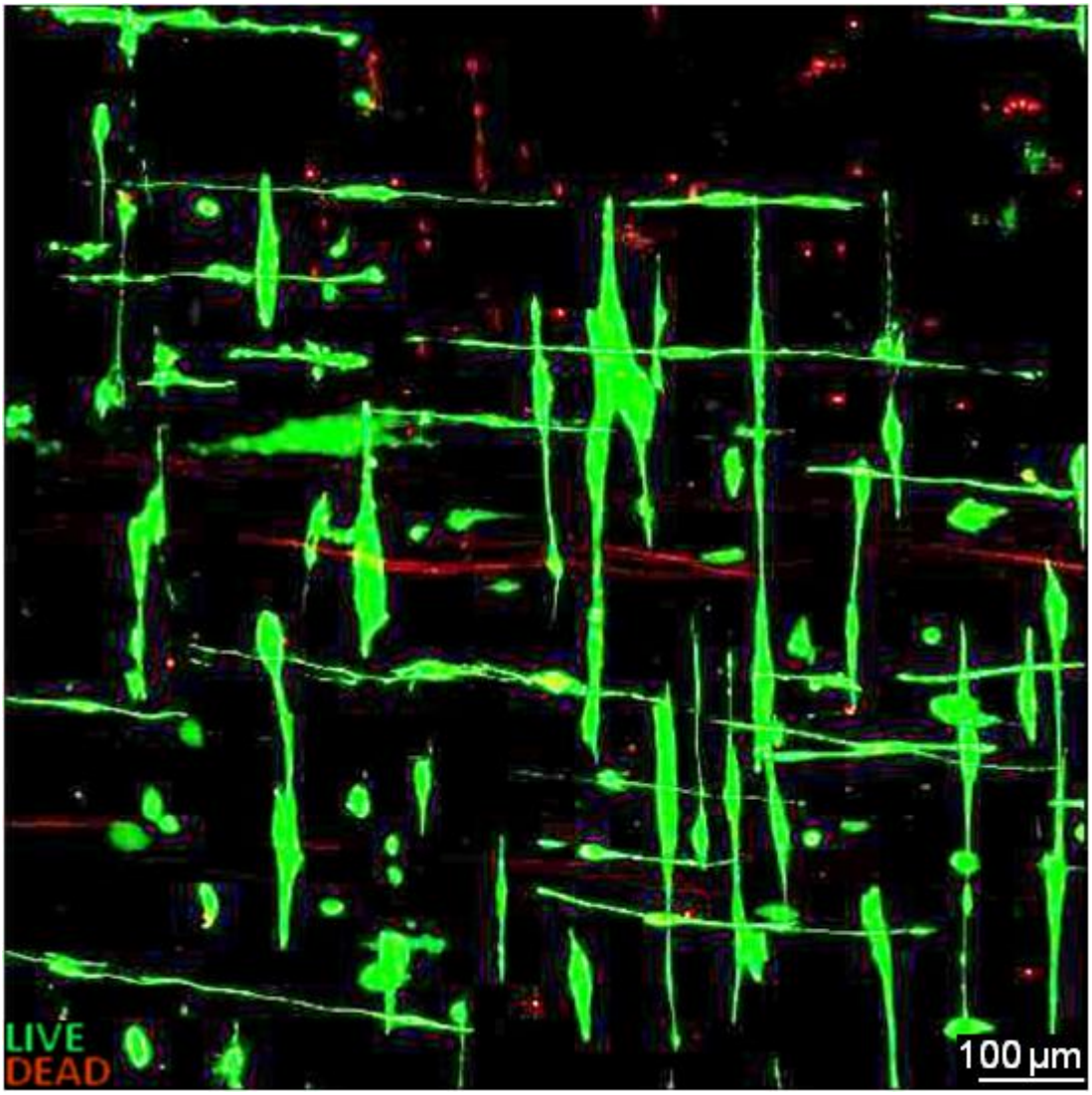
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