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Enhanced Neural Differentiation Using Simultaneous Application of 3D Scaffold Culture, Fluid Flow, and Electrical Stimulation in Bioreactors

*Simon Grossemy, Peggy P. Y. Chan, and Pauline M. Doran**

Neural differentiation is studied using a simultaneous application of 3D scaffold culture and hydrodynamic and electrical stimuli in purpose-designed recirculation bioreactors operated with continuous fluid flow. Pheochromocytoma (PC12) cells are seeded into nonwoven microfibrous viscose-rayon scaffolds functionalized with poly-L-lysine and laminin. Compared with the results from static control cultures with and without electrical stimulation and bioreactor cultures with the fluid flow without electrical stimulation, expression levels of the differentiation markers β3-tubulin, shootin1, and ephrin type-A receptor 2 are greatest when cells are cultured in bioreactors with fluid flow combined with in-situ electrical stimulus. Immunocytochemical assessment of neurite development and morphology within the scaffolds confirm the beneficial effects of exposing the cells to concurrent hydrodynamic and electrical treatments. Under the conditions tested, electrical stimulation by itself produces more pronounced levels of cell differentiation than fluid flow alone; however, significant additional improvements in differentiation are achieved by combining these treatments. Fluid flow and electrical stimuli exert independent and noninteractive effects on cellular differentiation, suggesting that interference between the mechanisms of differentiation enhancement by these two treatments is minimal during their simultaneous application. This work demonstrates the beneficial effects of combining several different potent physical environmental stimuli in cell culture systems to promote neurogenesis.

1. Introduction

The broadly transformative power of the physical environment to regulate cell lineage commitment and differentiation is now well recognized. For tissue engineering and the creation of functional tissue constructs in vitro, providing cells with appropriate physical stimuli can be as important as supplying them with suitable biochemical agents and trophic factors. From an initial focus on how properties of the attachment matrix direct cell behavior,[1] advances in our appreciation of cellular bioelectric and mechanotransduction processes have led to increasing interest in the use of additional physical stimuli, such as electrical, hydrodynamic, and applied mechanical forces, to control cell activity.[2,3]

Nerve tissue engineering based on the culture and neural differentiation of cells holds particular promise for the treatment of brain, spinal, and peripheral nerve injury and disease.[4] Recent studies have demonstrated the effects of several physical environmental conditions on nerve differentiation and development. Neural stem and progenitor cells, neurons, and glial cells are known to respond to the topography and surface properties of materials for modulation of cytoskeletal tension, gene activity, and cellular function (reviewed in ref. [5]). Such interactions are generally considered to underpin the practical observation that neural differentiation is often enhanced in 3D culture systems compared with traditional 2D monolayer cultures.[6,7] Electrical stimulation of cells has also received considerable attention as a strategy for enhancing neurogenesis in vitro (reviewed in ref. [8]). While much of this research has focused on developing electroconductive surfaces for 2D cell culture, electroactive 3D scaffolds have also been used to stimulate neural differentiation (reviewed in ref. [9]).

The relationship between externally applied mechanical forces and differentiation has so far been investigated mainly for cartilage, bone, and cardiac cells[10] rather than nerve cells. Yet, hydrodynamic shear stresses and the forces associated with subsonic vibration have been found to enhance the specification and development of neural cells on 2D and micropatterned 2D substrates.[11] Research into neurogenic responses to mechanical stimulation by cells in 3D cultures has been limited; however, a few studies of neural differentiation in scaffolds in the presence of fluid flow have been reported.[6,12] Despite the recognized value of mechanical and electrical stimuli in regulating cell differentiation in many tissues, little consideration has been given to the combined effects of these forces, especially for neurogenesis.

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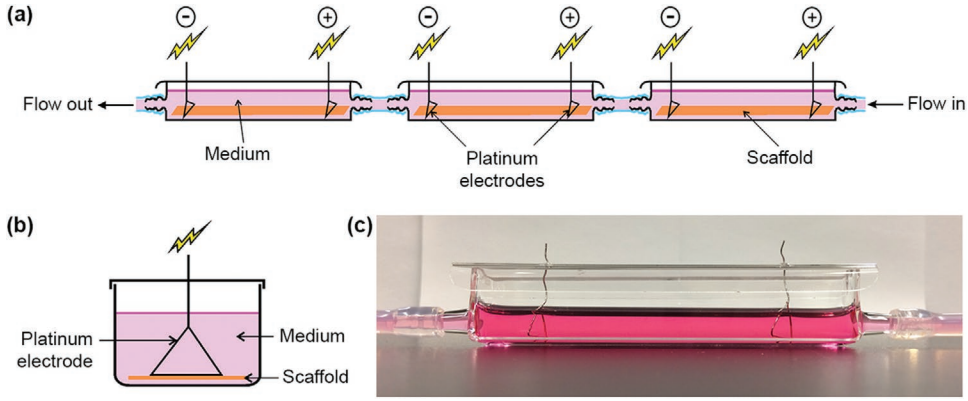

Figure 1. Recirculation bioreactor system. a) Schematic diagram of three bioreactor chambers connected in series and operated with medium flow. Cell-seeded scaffolds were placed in the chambers and fixed into position using two platinum wires that served as electrodes for electrical stimulation. Medium was recirculated through the bioreactor chambers using peristaltic pumps. b) Schematic end view of a single bioreactor chamber. c) Photograph of a single glass bioreactor chamber containing culture medium showing the platinum wire electrodes for electrical stimulation and silicone tubing connections for medium recirculation.

In our previous work, we reported the design and operation of a recirculation bioreactor suitable for culturing neural cells in 3D scaffolds in the presence of continuous fluid flow.[6] Using this bioreactor, rat adrenal pheochromocytoma (PC12) cells were exposed to hydrodynamic shear forces over an extended culture period for enhanced cell growth and neural differentiation without cell damage. In the present study, we adapted this device to allow simultaneous electrical and fluid mechanical stimulation of cells while maintaining the complex geometrical and topographical environment of 3D scaffold culture. Such a combination of physical stimuli has not previously been examined for neural cell differentiation. The response of cell cultures to this novel superimposition of forces was evaluated using gene expression and immunocytochemical analysis of key markers of neural lineage commitment, differentiation, and morphogenesis. Interaction analysis was also carried out to identify whether electrical and hydrodynamic treatments functioned independently, synergistically, or antagonistically during their concurrent application to cells.

2. Results

The bioreactor system of **Figure 1** was applied for cell culture in scaffolds with continuous fluid flow for up to 12 d, with or without electrical stimulation. The system operated in a stable fashion and maintained aseptic conditions for the duration of the cultures. Cells in scaffolds were also cultured for up to 12 d under static conditions without fluid flow, with or without electrical stimulation. The fluid flow and electrical conditions applied in this work were determined previously to have no detrimental effect on cell viability or cell attachment to the scaffolds.[6,13]

Results from quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis of nestin, β3-tubulin, shootin1, and ephrin type-A receptor 2 (EphA2) gene expression are shown in **Figure 2**. For each marker and culture duration, transcript levels are presented relative to those for the static cultures without electrical stimulation. The results for nestin, a marker of neural stem and progenitor cells,[14] showed no significant difference between the treatment groups after either 6 or 12 d of culture (Figure 2a). Expression of β3-tubulin, a structural cytoskeletal component essential for neurite outgrowth and morphogenesis,[15] was significantly greater ($p < 0.01$) in the bioreactor cultures with electrical stimulation compared with all other treatment groups, after both 6 and 12 d of culture (Figure 2b). Similar results at either the $p < 0.01$ or $p < 0.05$ level of significance were found for shootin1, which is involved in neuronal polarization and axon induction in early-stage neuritogenesis,[16] and the receptor, EphA2, which regulates neural lineage commitment, cell adhesion, and axon guidance[17] (Figure 2c,d). Overall, the qRT-PCR results indicate that more pronounced neural differentiation was achieved using bioreactors with combined fluid flow and electrical stimulation compared with static cultures without electrical stimulation, static cultures with electrical stimulation, and bioreactor cultures without electrical stimulation.

Images from immunocytochemical staining of β3-tubulin and microtubule associated protein 2 (MAP2) after 6 and 12 d of culture are shown in **Figure 3**. MAP2 binds to several cytoskeletal elements and plays an important role in neuronal outgrowth and remodeling.[18] Because MAP2 is detected later during embryogenesis than β3-tubulin, it is generally considered to indicate more advanced neuronal differentiation.[19] In the bioreactor cultures with electrical stimulation, neurite extensions had started to form after 6 d of culture (Figure 3a) but were much longer and had developed more complex structures and networks after 12 d (Figure 3b). For comparison, immunocytochemistry results after 12 d of other treatments are also shown in Figure 3; additional images for static cultures with and without electrical stimulation were reported previously.[13] Combined fluid flow and electrical stimuli in the bioreactors (Figure 3b) generated denser and more abundant neurite networks than in either the bioreactor cultures without electrical stimulation (Figure 3c) or the static cultures with electrical stimulation (Figure 3d). Because of the masking and foreshortening effects inherent in the fibrous 3D scaffolds, accurate quantification of neurite number and length could

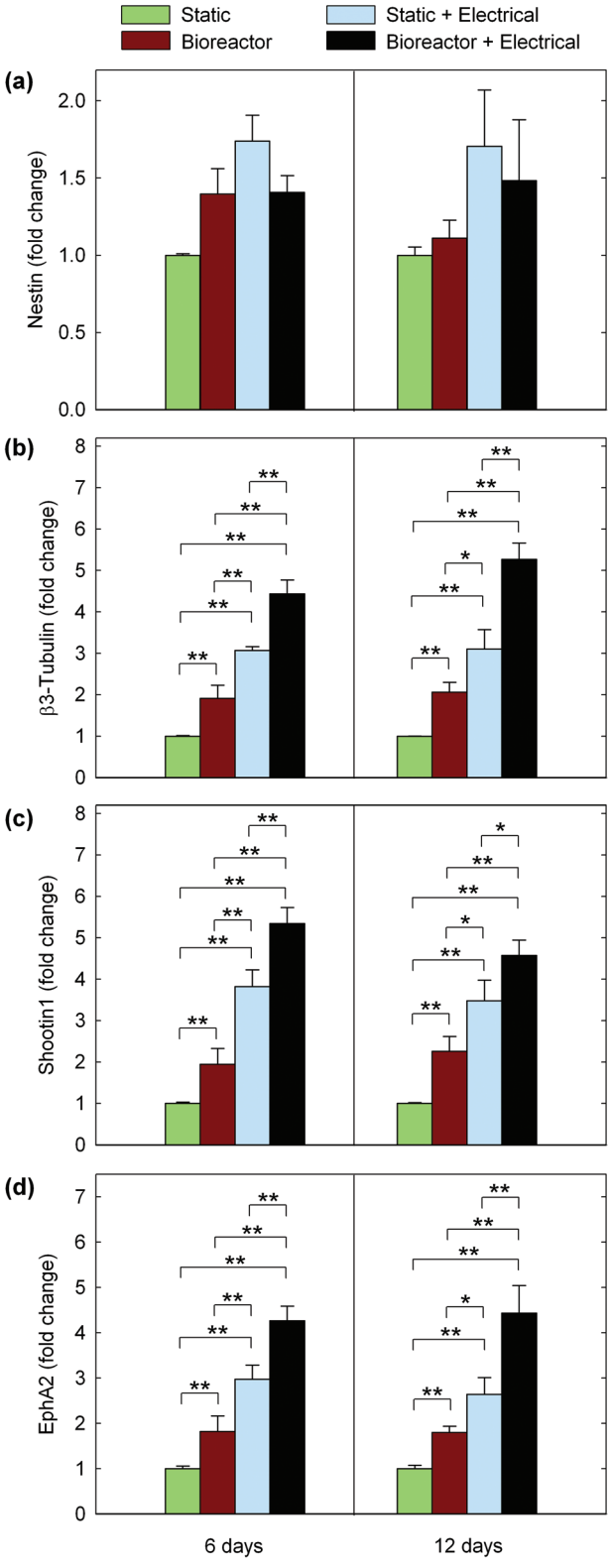

Figure 2. Gene expression levels for: a) nestin, b) β3-tubulin, c) shootin1, and d) EphA2. Results are shown for static cultures without fluid flow and without electrical stimulation (static); bioreactor cultures with fluid flow without electrical stimulation (bioreactor); static cultures without fluid flow with electrical stimulation (static + electrical); and bioreactors with combined fluid flow and electrical stimulation (bioreactor + electrical). The cells were cultured for either 6 or 12 d. For each marker and culture duration, the results are presented relative to the transcript levels for static cultures. The error bars represent standard errors from triplicate cultures ($n = 3$). Significant differences at the $p < 0.05$ and $p < 0.01$ levels determined using one-way ANOVA and Tukey's test are indicated * and **, respectively.

not be carried out from visual observation using the images of Figure 3. Overall, the qualitative immunochemistry results of Figure 3 confirm the findings obtained from gene expression analysis (Figure 2).

The more advanced level of differentiation achieved when fluid flow and electrical treatments were combined (Figures 2 and 3) indicate that the positive effects on cells of these two different stimuli were accumulative during their simultaneous application. To determine which, if any, of these two treatments exerted a dominant influence on neural differentiation, and to identify any interactive effects between them, the data from Figure 2 for gene transcript levels after 6 and 12 d of differentiation are presented in **Figure 4** as interpretation graphs. Increases in gene expression for nestin, β3-tubulin, shootin1, and EphA2 are plotted in percentage terms relative to static conditions without electrical treatment. In each graph, the effect of electrical stimulation is identified using two different colors for the datum points, viz. blue (without electrical stimulation) and red (with electrical stimulation). The effect of fluid flow is represented by separation of the data along the horizontal axis of each graph for static (without fluid flow) and bioreactor (with fluid flow) culture conditions. The positive slopes of all lines in the plots for β3-tubulin, shootin1, and EphA2 (Figure 4c–h) reflect the beneficial effect of fluid flow in the bioreactors on expression of these markers, irrespective of the presence or absence of electrical stimulus. The position of the data shown in red for cultures with electrical stimulation above the data shown in blue for cultures without electrical stimulation reflects the greater beneficial effect on neural differentiation of electrical stimulus compared with fluid flow. For all differentiation markers except nestin, the blue and red lines on the graphs are close to parallel (Figure 4c–h), indicating little or no interaction between fluid flow and electrical stimulation in their effects on neural differentiation. These observations derived from the patterns of gene expression for β3-tubulin, shootin1, and EphA2 did not apply to nestin (Figure 4a,b), suggesting a general lack of correlation between nestin expression and neurogenic development. The overall conclusion from Figure 4 is that the positive effects of fluid flow and electrical stimulation on neural differentiation were largely independent of each other, i.e., the effects of one treatment were neither enhanced nor diminished in a major way when combined with the other treatment.

3. Discussion

Simultaneous application of fluid flow and electrical stimuli to cells in 3D scaffolds requires development of a suitable bioreactor device capable of aseptic operation for aerobic cell culture

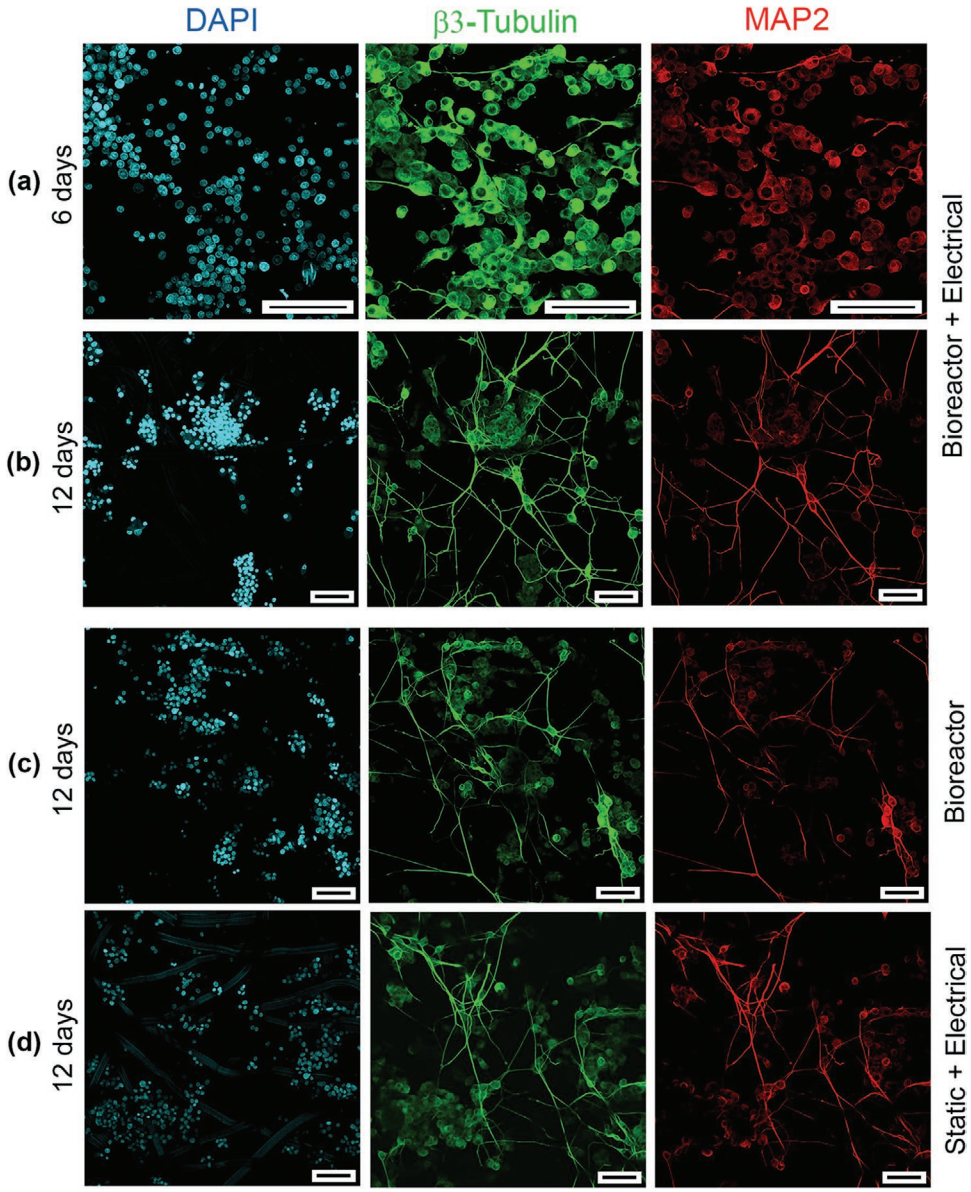

Figure 3. Confocal microscope images of cells in scaffolds cultured in: a,b) bioreactors with combined fluid flow and electrical stimulation (bioreactor + electrical); c) bioreactors with fluid flow without electrical stimulation (bioreactor). Reproduced with permission.[6] Copyright 2020, Elsevier; and d) static cultures without fluid flow with electrical stimulation (static + electrical). The cells were cultured for a) 6 d or b–d) 12 d. Samples were stained for DAPI (blue), β3-tubulin (green), and MAP2 (red). All scale bars represent 100 µm.

over several days or weeks. In this work, a recirculation bioreactor system with capacity for electrical stimulation of cells in microfibrous 3D scaffolds (Figure 1) was operated with continuous fluid flow under stable conditions for 12 d. The electrical stimulation and bioreactor operating conditions employed were the same as those identified in previous work to provide a non-cytotoxic, nondamaging environment for PC12 cell culture.[6,13] Immortalized PC12 cells have been applied extensively as a well characterized model for studying the effects of external stimuli on neurogenesis.[20,21] For the development of medical technology suitable for application to humans, further in-vitro and in-vivo experimentation is required using other cell types and/or animal systems.

To our knowledge, the combined effect of fluid flow and electrical stimulation on neural differentiation has not been reported previously using either 2D or 3D culture geometry. Sagita et al.[22] described the fabrication of a microfluidic bioreactor capable of providing electrical stimulation; however, that system was scaffold-free and thus presumably suitable for 2D culture only, and no biological data or cellular responses to the treatment conditions were evaluated. In the present study, quantitative and qualitative assessment of several differentiation markers demonstrated that the combination of fluid flow and electrical stimulation produced significant benefits for neural differentiation in 3D scaffolds compared with fluid flow or electrical treatment alone (Figures 2–4). Our culture system

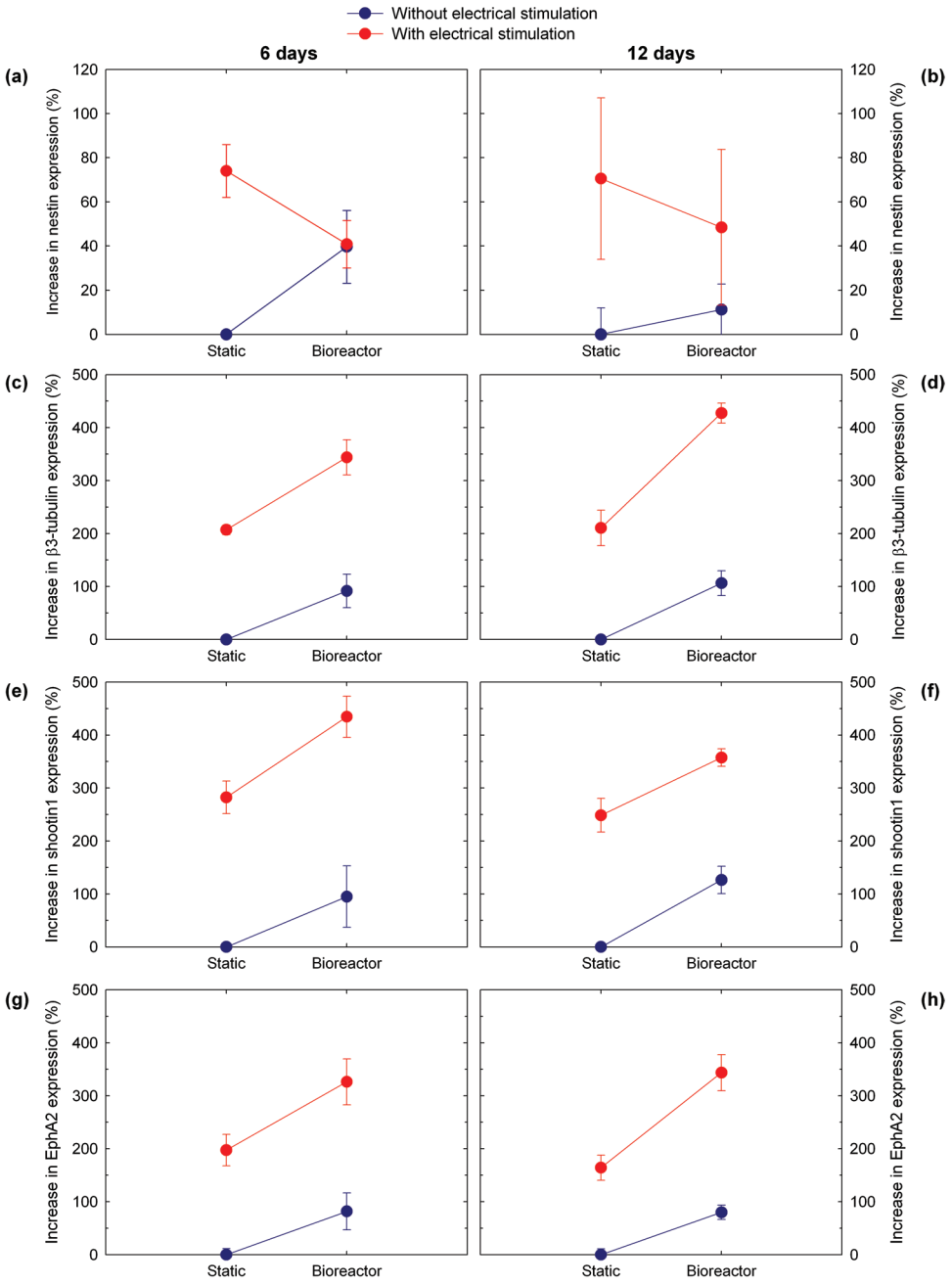

Figure 4. Graphs for determining the interaction of factors based on gene expression results after 6 and 12 d of culture for a,b) nestin; c,d) β3-tubulin; e,f) shootin1; and g,h) EphA2. The error bars represent standard errors from triplicate cultures ($n = 3$). In each graph, data for cultures without electrical stimulation are shown in blue; data for cultures with electrical stimulation are shown in red. The effect of fluid flow is represented along the horizontal axis of each graph for static (without fluid flow) and bioreactor (with fluid flow) culture conditions. The positive slopes of most lines reflect the beneficial effect of fluid flow in the bioreactors, with or without electrical stimulation. In each graph, the higher values of the data shown in red for cultures with electrical stimulation reflect the greater beneficial effect on neural differentiation of electrical stimulus compared with fluid flow. Most pairs of blue and red lines in each graph are close to parallel, indicating little or no interaction between fluid flow and electrical stimuli in their effects on neural differentiation.

thus offers potential practical advantages for promoting differentiation and regeneration of nerve cells and tissues in vitro. The positive effects of simultaneous hydrodynamic and electrical stimulation of cells were demonstrated using a constant, fixed fluid flow rate and a single electrical potential difference. Further enhancements in cell and tissue development may be achieved by varying the culture conditions over a wider range of values and combined environmental settings than those tested here.

Electrical stimulation by itself produced a quantitatively greater beneficial effect on neural differentiation than bioreactor culture alone; however, substantial further improvements

were achieved by applying these stimuli simultaneously (Figures 2–4). Whereas positive responses were obtained in terms of β3-tubulin, shootin1, and EphA2 expression (Figure 2b–d), there was no clear trend in nestin transcript levels following either flow or electrical stimulation or a combination of these treatments (Figure 2a). Variation between replicate samples was also relatively greater for nestin than for the other differentiation markers. Nestin is an intermediate filament protein present during early embryonic development and is closely associated with cell proliferation and the undifferentiated status of stem and neural precursor cells.[14] Because its expression reduces rapidly as cells progress toward differentiation, the results for nestin observed in this work likely reflect endogenous fluctuations in expression of this protein during transition of cells from early neural commitment to a more advanced stage of neural differentiation.

Cell behavior is regulated by fluid flow through enhancement of external mass transfer processes and/or by activation of biological mechanotransduction mechanisms that convert exogenous mechanical stimuli into cytoskeletal tension and component binding. Molecular and structural changes in the cytoskeleton trigger intracellular kinase cascades, such as the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathways, that transmit signals to the cell nucleus for induction of gene expression and differentiation.[2,23] Rapid activation of mechanosensitive ion channels and mobilization of second messengers such as Ca^{2+} are also fundamental elements of the biochemical response of cells to fluid flow.[23,24] Neural cells conform to this typical regulatory programme: for example, neural stem cells in the brain react to the flow of cerebrospinal fluid by modulating ion-channel activity, Ca^{2+} signaling, and ERK phosphorylation for control of neurogenesis.[25] These same biochemical processes are also used by cells in their response to electrical stimulation. Exogenous electrical fields have the potential to influence neurogenesis through cytoskeleton reorganization, specific ion-channel activation, and Ca^{2+} transients that can trigger intracellular signaling cascades; additional responses to electrical stimulus such as polarization of plasma membrane structures, redistribution of charged cell-surface receptors, and directional cell migration may also be deployed to control neural development.[8] In the present study, fluid flow and electrical stimulation were found to have largely independent beneficial effects on neural differentiation (Figure 4c–h). These results suggest that the mechanisms of differentiation enhancement for the two treatments were sufficiently different and closely regulated that they did not interfere with each other or, if the same mechanism was elicited, its processes were not saturated by either stimulus alone under the conditions tested. Simultaneous application of fluid flow and electrical stimulation to cells in 3D culture thus has considerable potential utility for the advancement of nerve tissue engineering.

4. Conclusion

We report the design and operation of a simple recirculation bioreactor system suitable for exerting combined hydrodynamic and electrical stimuli on cells in 3D scaffolds. Compared with static 3D cultures, neural differentiation was enhanced by treatment of PC12 cells with fluid flow alone, electrical stimulation alone, or flow and electrical stimuli combined. Under the conditions tested, electrical stimulation by itself was more beneficial than fluid flow; however, combination of these treatments produced significant further improvements in neural differentiation. Analysis of the interactive effects of fluid flow and electrical treatment indicated that these two stimuli enhanced cellular differentiation independently and did not markedly interfere with each other when applied in combination.

5. Experimental Section

Cell Culture: PC12 cells were maintained in growth medium comprising Dulbecco's modified Eagle's medium containing glucose (4.5 g L^{-1}), L-glutamine (584 mg L^{-1}), and sodium bicarbonate (3.7 g L^{-1}) (DMEM), with horse serum (10%), fetal bovine serum (5%), and penicillin-streptomycin antibiotic solution (1%), as described previously.[13] The differentiation medium used in the experiments was DMEM with horse serum (1%), penicillin-streptomycin solution (1%), and nerve growth factor (NGF-7S) (100 ng mL^{-1}). NGF was added fresh to the medium 1 h before its use.

Scaffolds and Cell Seeding: The scaffold material was nonwoven microfibrous viscose-rayon derived from plant cellulose and purchased from Synergy Health (Swindon, UK) in the form of Azowipettes. The scaffold matrix had an open fibrous structure with average fiber diameter 20 μm, porosity 66%, and pore sizes of 50–300 μm[6,13] (**Figure 5**a). Scaffolds of size 7 cm × 1 cm were sterilized by autoclaving, coated with poly-L-lysine and laminin, and seeded with PC12 cells at a density of 10^5 cells per cm^2 of scaffold superficial area, as described previously.[13] The unseeded scaffolds and the poly-L-lysine and laminin coatings were nonconductive; the culture medium displayed nonohmic behavior typical of electrolyte solutions with a resistance of 6.1×10^4 Ω at 500 mV measured in Petri dishes.[13] After seeding, the scaffolds were incubated in growth medium for 2 d before being transferred to differentiation medium for a further 2 d prior to the experiments. Cells after these preculture steps were considered to be at time zero: durations of the static and bioreactor cell cultures were measured in days after this point.

Unlike cells cultured on 2D surfaces, cells in the 3D scaffolds were able to fully surround the scaffold fibers and thus grow in all directions (Figure 5b); they also exhibited rounded rather than flat morphology. By providing a structurally heterogeneous culture environment, fibrous 3D scaffolds offer cells a more diverse range of micromechanical and mechanotransductional conditions than in 2D surface culture, reflecting the variations in size, orientation, and spatial density of the matrix fibers, and the nonuniform distribution of adhesion and extension forces as individual cells interact with the fibers and each other in 3D.[26] The ability of cells to develop dense and elaborate neural networks is also significantly enhanced using 3D rather than 2D geometry, as indicated in Figure 5c which illustrates the extension of neurital processes and connectivity between cells across different depths within the scaffold.

Bioreactor: The recirculation bioreactor system used for culture of the cells is described in detail in ref. [6]. The main features of the apparatus are shown in Figure 1. In each bioreactor assembly, a medium reservoir was connected to three individual glass bioreactor chambers arranged in series. Each bioreactor chamber contained one 7 cm × 1 cm cell-seeded scaffold that was fixed into position using two platinum wires (0.5 mm diameter, 99.9% purity, annealed: Goodfellow, UK) attached to the chamber lid. The bioreactor chambers were operated inside an incubator at 37 °C, 5% CO_2, and 95% humidity. Differentiation medium was recirculated through the bioreactor assembly using peristaltic pumps at a volumetric flow rate of 0.51 mL min^{-1} corresponding to laminar flow with a Reynolds number of 1.1 and estimated fluid-induced shear stress levels of 2.7×10^{-5} to 4.6×10^{-3} Pa.[6] Although up to three bioreactor chambers in series could be operated concurrently

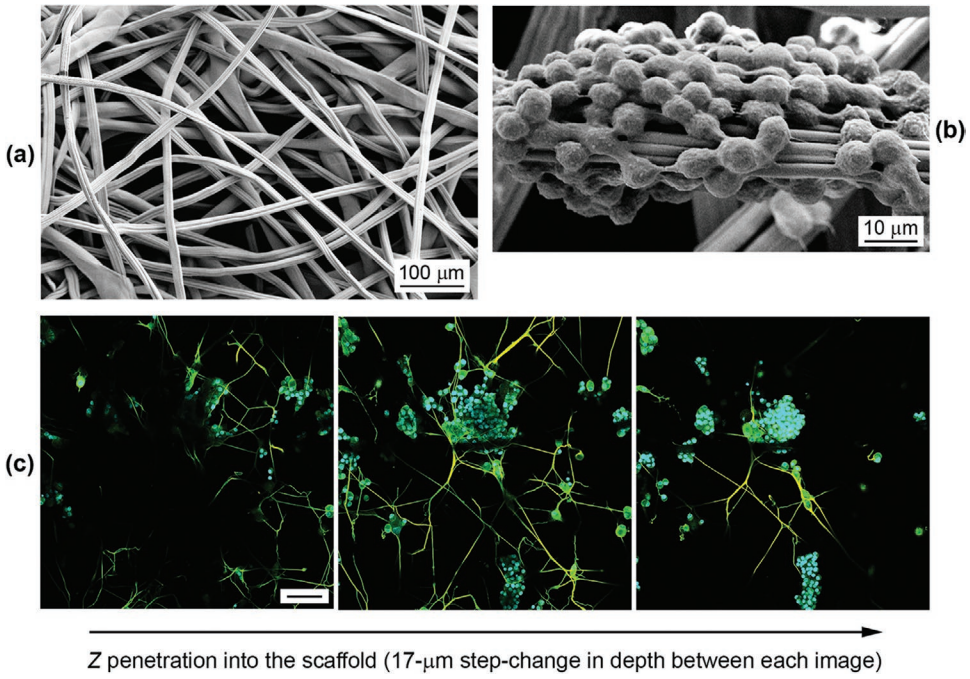

Figure 5. a) Scanning electron image of a microfibrous scaffold after coating of the fibers with poly-L-lysine and laminin. b) Scanning electron image of a scaffold seeded with cells after 6 d of culture in growth medium. c) Confocal microscope images of cells in a scaffold showing merged staining for DAPI, β3-tubulin, and MAP2. The cells were cultured for 12 d in bioreactors with combined fluid flow and electrical stimulation. From left to right, the images in (c) show neurital extensions and connections at increasing penetration or depth (*Z*) within the scaffold, each image in the series denoting a step change in penetration of 17 μm. The scale bar in (c) represents 100 μm and applies to all three images in (c).

in a single bioreactor assembly as shown in Figure 1, such a single apparatus was not considered to provide triplicate cultures for statistical analysis because each bioreactor chamber in the assembly was not operated independently. To obtain triplicate bioreactor samples, three independent recirculation bioreactor assemblies, each with three bioreactor chambers, were used. For each time point of analysis, triplicate data were derived from three bioreactor chambers, one from each of the three independent bioreactor assemblies. When any individual chamber had to be removed for analysis, or when medium exchange was required, each entire bioreactor circuit was brought into a biosafety cabinet to avoid contamination of those parts of the bioreactor system remaining in operation.

Static and Bioreactor Cell Cultures: Static cell cultures were carried out for 6 or 12 d in glass Petri dishes at 37 °C, 5% CO_2, and 95% humidity with and without electrical stimulation as described previously.[13] Partial (50%) medium replacement was carried out every 2 d. For electrical stimulation of static cultures, a DC electric field was applied across triplicate scaffolds immersed in differentiation medium in the Petri dishes using a signal generator and platinum wire electrodes. A fixed potential difference of 500 mV (8.3 mV mm^{-1}) was used; the current generated under these conditions was 8–9 μA. Electrical stimulation was applied for 2 h each day.

Bioreactor cultures with and without electrical stimulation were carried out under constant flow conditions for 6 or 12 d. Partial (50%) medium replacement was carried out every 2 d. To combine the effects of medium flow and electrical stimulation, 500 mV was applied across each scaffold in the bioreactors by connecting the platinum wires attached to the lids of the bioreactor chambers to the signal generator. Electrical stimulation in the bioreactors was applied for 2 h each day.

Imaging and Analyses: Scanning electron microscopy was used for scaffold imaging as described previously.[13] The differentiation status of the cells was assessed after 6 and 12 d of culture. qRT-PCR was used to measure gene transcript levels as described previously.[13] Expression of nestin, β3-tubulin, shootin1, and EphA2 was examined by qRT-PCR using ribosomal protein L19 (RPL19) as the housekeeping gene. Cells within the scaffolds were labeled with 4′,6-diamidino-2-phenylindole (DAPI) to locate cell nuclei, immunocytochemically stained for β3-tubulin and MAP2, and examined using confocal laser-scanning microscopy as described previously.[13]

Statistical Analysis: All quantitative data were subjected to statistical analysis. Samples were harvested from triplicate cultures ($n = 3$). The measured data are presented as averages ± standard errors. Treatment groups were compared using one-way analysis of variance (ANOVA) in conjunction with Tukey's test at $p < 0.05$ and $p < 0.01$ levels of significance.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

bioreactors, electrical stimulation, microfibrous scaffolds, neural differentiation, PC12 cells

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