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A miniaturized bioreactor system for the evaluation of cell interaction with designed substrates in perfusion culture

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Abstract

In tissue engineering, the chemical and topographical cues within three-dimensional (3D) scaffolds are normally tested using static cell cultures but applied directly to tissue cultures in perfusion bioreactors. As human cells are very sensitive to the changes of culture environment, it is essential to evaluate the performance of any chemical, and topographical cues in a perfused environment before they are applied to tissue engineering. Thus the aim of this research was to bridge the gap between static and perfusion cultures by addressing the effect of perfusion on cell cultures within 3D scaffolds. For this we developed a scale down bioreactor system, which allows to evaluate the effectiveness of various chemical and topographical cues incorporated into our previously developed tubular ϵ -polycaprolactone scaffold under perfused conditions. Investigation of two exemplary cell types (fibroblasts and cortical astrocytes) using the miniaturized bioreactor indicated that: (1) quick and firm cell adhesion in 3D scaffold was critical for cell survival in perfusion culture compared with static culture, thus cell seeding procedures for static cultures might not be applicable. Therefore it was necessary to re-evaluate cell attachment on different surfaces under perfused conditions before a 3D scaffold was applied for tissue cultures, (2) continuous medium perfusion adversely influenced cell spread and survival, which could be balanced by intermittent perfusion, (3) micro-grooves still maintained its influences on cell alignment under perfused conditions, while medium perfusion demonstrated additional influence on fibroblast alignment but not on astrocyte alignment on grooved substrates. This research demonstrated that the mini-bioreactor system is crucial for the development

of functional scaffolds with suitable chemical and topographical cues by bridging the gap between static culture and perfusion culture.

Key words: Miniaturized bioreactor, perfusion culture, astrocytes, fibroblasts, tissue engineering.

1. Introduction

It has been well established that macro-, micro-, and nanoscale patterns of chemistry and topography play important roles in controlling cell behaviours (Chen *et al.*, 1997; Curtis and Wilkinson 1997; Flemming *et al.*, 1999; Stevens, George, 2005) depending on different cell sensitive scales (Massia and Hubbell 1991; Britland *et al.*, 1996; Wojciak-Stothard *et al.*, 1996; Curtis and Wilkinson 2001; Anderson *et al.*, 2003). Thus considerable research efforts have been focused on the development of three-dimensional (3D) tissue engineering scaffolds for optimal cell and tissue growth (Hubbell, 2003; Stevens and George, 2005; Zong *et al.*, 2005). Previously we fabricated tubular constructs (coined ‘Swiss roll’) with potential applications in vascular and nerve tissue engineering (Gadegaard, *et al.*, 2008. Seunarine, *et al.*, 2008). The Swiss rolls were made of a thin (<50 µm) biodegradable ε-polycaprolactone (PCL) (Robert *et al.*, 1993) membrane with nano- and/or microtopographies on either (both) side(s) to guide, promote or inhibit specific cellular responses (Clark *et al.*, 1987; Casey 1999; Csaderova *et al.*, 2010).

Apart from controlling cell behaviours using the chemical and topographical cues, the success of tissue engineering is also dependent on the maintenance of nutrient throughout the scaffold after cell colonization (Salem *et al.*, 2002; Sun *et al.*, 2005). Due to the limited mass diffusion between the bulk medium outside the scaffold and the cells within the majority of reconstructed tissues (Terai *et al.*, 2002), the depletion of medium nutrients and accumulation of toxic waste products associated with static culture will adversely affect cell survival. There are also other issues with the long-term static culture of tissue-engineered constructs, as this requires repetitive handling (e.g., growth medium replenishment every few days), which can increase the risk of contamination. For the preparation of metabolically demanding tissues within 3D scaffolds at relatively large scale, medium perfusion is thus likely to be more effective than frequent changes of static culture medium, as it will not only support metabolic activity at a higher level compared to static culture (Jelinek *et al.*, 2000; Link *et al.*, 2004), but also reduce the risk of contamination caused by repetitive handling. However,

perfusion culture is different from static culture as cells are exposed to medium flow and thus the associated shear force, the constant local disequilibrium etc, while it is well established that human cells are extremely sensitive to differences in culture environments (Sun *et al.*, 2004). Therefore, it is crucially important to test the biological performances of such scaffolds not only in static-culture, but also in perfusion-culture. However, it was almost impossible to evaluate the performances of chemical/topographical cues within the Swiss rolls using currently available live cell imaging technologies during tissue culture due to its multilayered tubular structure, layers of translucent membranes and large size. Although conventional cell analysis methods such as biochemical analysis, histology, and or immuno-staining can be used *post-hoc* at predetermined time-points, the tissues have to be sacrificed. The fragile cell structures inside the scaffold could be damaged during fixation, embedding with highly viscous media, cutting and staining, and thus vital information about cell morphology and distribution within different parts of the scaffold might be lost. Thus, the aim of this study was to develop and evaluate a scale down bioreactor system with multiple mini-chambers in a 6-well plate, which would allow to test different chemical and/or topographical cues under perfused conditions, before the engineered scaffolds will be used for tissue engineering. In order to maintain the generality and transferability of this miniaturised bioreactor system, and also because of the potential application of the Swiss rolls to various areas of tissue engineering, two different cell types were selected to evaluate the bioreactor system. As one of the major supportive glial cell types in the central nerve system (Noble and Murray, 1984; Sørensen *et al.*, 2007), type 1 cortical astrocytes were selected as an exemplary cell type that would encounter such structures in central nervous tissue engineering. hTERT fibroblasts were selected because fibroblasts play important roles in structure formation, and various wound healing processes (Gailit and Clark 1994; Mirastschijski *et al.*, 2004). In this research, the bioreactor system was not only used to evaluate various chemical and topographical cues on the behaviours of two cell models, but also to optimise the bioprocess for perfusion cultures. Our research indicated that the mini-bioreactor system could

be used as an *in vitro* simulator to investigate various complex issues in tissue engineering, which will allow to bridge the gap between static cell cultures on substrates with surface microstructures (2.5D substrates) and perfused tissue cultures with 3D scaffolds.

2. Materials and Methods

2.1. Cell Culture

As described previously (Noble and Murray 1984; Lakatos *et al.* 2000) purified type 1 cortical astrocytes were prepared by first digesting cortices (dissected from 1-day old Sprague Dawley rats) in 1.33% collagenase (Sigma, Poole, UK), seeding ($\sim 2 \times 10^7$ cells per T75 flask) and culturing the cells in a poly-L-lysine coated T75 flask for 10-12 days. The cells were maintained in DMEM (Invitrogen, Paisley, Scotland) supplemented with 10% foetal bovine serum (FBS) (Invitrogen, Paisley, Scotland) and L-glutamine (2mM, Sigma). Confluent flasks were shaken on a rotary platform overnight at 37°C to remove contaminating oligodendrocyte precursor cells. The remaining cells after this procedure were 85-95% type 1 cortical astrocytes as judged by labelling for glial fibrillary acidic protein (GFAP), a cell-type specific marker for astrocytes. The astrocytes were passaged no more than 4 times and detached using trypsin/EDTA (0.02% solution) for experiments when almost 100% confluent. hTERT fibroblasts (immortalised from primary human BJ foreskin fibroblasts, Clontech Laboratories, Inc. USA) were cultured using the same medium as cortical astrocytes and detached for experiments when approximately 90% confluent.

2.2. Fabrication and treatment of micro-grooved PCL substrates

Micro-grooved PCL (Mw 65 000, CAS 24980-41-4, Aldrich, Poole, UK) substrates were prepared by hot embossing as described by Gadegaard *et al.* (2003). Apart from the micro-grooves for cell alignment, rows of pillars at a relatively larger scale were also employed, these were incorporated to maintain space between multiple layers of PCL substrate within the Swiss-roll to facilitate cell

growth and to some extent medium diffusion (Gadegaard *et al.* 2003, 2008; Seunarine *et al.* 2008). Thus three types of PCL substrates were prepared and investigated in this study: (A) grooved/pillared PCL substrate containing an alternating pattern of parallel grooves and ridges with the same width (25 μm) and depth (5.0 μm), and rows of pillars (pillar size: 50 μm wide, 400 μm long, 50 μm high, distance between pillars in each row: 100 μm , row to row distance: 400 μm), (B) pillared PCL substrates containing only rows of pillars (pillar dimension and spacing as described above), and (C) flat PCL substrates without any micro-topographies. Prior to experimentation all the substrates were sterilized with 70% ethanol for 24h, rinsed thoroughly with sterilized reverse osmosis (RO) water, dried and then treated using various surface coating methods: poly-L-lysine coating (PLL): incubated with 13 $\mu\text{g}/\text{ml}$ poly-L-lysine (Sigma, Dorset, UK) for 1-12 h and then washed thoroughly with phosphate-buffered saline (PBS). Collagen coating (COL): coated with collagen solution (APCOLL-S, Purified Soluble Collagen, a gift from Devro Medical Limited, Glasgow, UK, 3.1mg/ml) for 1–12 h and then washed thoroughly with PBS. Plasma treatment (P): treated with a Harrick Plasma Cleaner (Harrick Plasma, Ithaca, NY, USA) at Hi settings (740V DC, 40 mA DC, 29.6W) for 5 minutes. Plasma treatment and PLL coating (P-PLL): first plasma treated then coated with PLL as described earlier. Plasma treatment and COL coating (P-COL): first plasma treated then coated with COL as described earlier. Non-treatment (NON): the PCL substrates were not treated or coated. All the treatment methods were assessed using two methods: measuring contact angle, and cell adhesion under static and perfused culture conditions. The effect of coating on hydrophobic or hydrophilic properties of the treated surfaces were analyzed using a commercially available contact angle measurement setup (CAM 100, KSV Instruments LTD, Finland), their influence on cell attachment, spreading, and survival was analyzed as described below.

2.3. Design and fabrication of the bioreactor system with multiple mini perfusion chambers

As illustrated in Figure 1A, the bioreactor system consisted mainly of two commercially available 6-well tissue culture plates situated in two plastic boxes (180mm long, 110mm wide, 60mm deep)

respectively, an 8-channel Ismatec peristaltic pump (IPC-N, ISMATEC, Switzerland), 8 medium reservoirs (2.5ml) and 3-way valves, all of which were connected with silicone tubes (Inside diameter: 1mm). Corresponding to the 8 channels of the peristaltic pump, 8 mini perfusion chambers were fabricated in the 6-well plates. Inside each of the mini perfusion chambers (Figure 1B to 1C), was a sandwich which started at the bottom with a piece of rectangular PCL substrate (15 mm × 30 mm × 0.15mm), followed by a circular silicone spacer (35mm in diameter, 1mm thick) with a rectangle chamber (5mm×25mm) created inside, then a circular transparent polystyrene plate (35mm in diameter, 1mm thick) with two small holes (2mm in diameter) that were 20mm apart, a circular silicone sealing plate (35mm in diameter, 4mm thick) with an observation window (approximately 10mm×10mm) inside and a plastic o-ring (outside diameter 35mm, inside diameter 25 mm). The PCL substrate, rectangle chamber inside the silicone spacer and the polystyrene plate together formed the mini perfusion chamber (25mm long, 5mm wide and 1mm deep) with two holes on both ends, which were sealed by the silicone sealing plate. The perfusion chamber was clamped and fixed within each well by the plastic o-ring. Two needles (18 G1^{1/2}"") were inserted into the perfusion chamber through the thick silicone sealing plate and the two holes on the polystyrene plate and used as inlet and outlet of the perfusion chamber respectively. Each perfusion chamber was connected to an individual channel of a peristaltic pump and a medium reservoir using gas permeable silicone tubes (Dow Corning, Seneffe, Belgium). These closed loops also contained 3-way valves, which were used to seed cells in the chamber and change medium in the reservoir if necessary. The medium reservoirs were also employed to capture air bubbles created in the tubing system during cell culture. After the perfusion chambers were set-up, the two 6-well plates were placed into the sterilised plastic boxes for subsequent cell seeding and culture experiments. Prior to experimentations, each perfusion system was sterilised by perfusing 70% (v/v) ethanol / 30% (v/v) sterile water overnight, washed with PBS for 60 minutes (at 1 ml/minute) and subsequently filled with cell culture medium. The perfusion chamber was then seeded with either astrocytes or

fibroblast suspension (approximately 5×10^5 cells/ml, or 500 cells/mm² of the substrate) introduced using a syringe through the 3-way valves (in order to bypass the other parts of the bioreactor system). The entire system was placed in a tissue culture incubator (37°C, 95% air/5% CO₂), thus the gas dissolved within the medium inside the closed loop system equilibrated with the gas-mix within the incubator through the gas permeable silicone tubes. After static incubation for different periods of time, to allow cell attachment to the PCL substrates, the cells were subjected to medium perfusion with varying flow rates (from 0.3ml/hour to 20ml/hour). Static control cultures were carried out in parallel to the perfused, depending on the experimental design. For all the static control culture experiments, the substrates were first incorporated into the mini bioreactor systems, seeded with cells, and then incubated statically (approximately for 45 min) to allow for cell attachment. The cell loaded substrates were then transferred from the mini chambers into separate 6-well plates for subsequent static culture. For comparison the static control was also referred to as: “perfused culture with 0 ml/h flow rate.

The equivalent shear rate was calculated using the following equation: $\tau_{wall} = 6\eta * Q/h^2w$ according to Lu et al. (2004) and Korin, et al. (2007), where ‘ τ_{wall} ’ is the shear stress at the walls, ‘Q’ represents the flow rate within the channel, ‘ η ’ is the viscosity, ‘h’ is the height of the channel and ‘w’ is the width of the channel.

During cell culture, the cells were monitored using either intermittent phase contrast microscopy or time-lapse microscopy on a Zeiss Axiovert 25 (Zeiss, Welwyn Garden City, UK) inverted phase contrast microscope connected to a video camera and image capture setup (Alrad Scientific, Newbury, UK) situated in a temperature and CO₂ controlled environmental chamber. After cell culture, perfusion chambers were disassembled and the cell/substrates removed for various analyses. All the components of the bioreactor system except the PCL substrates were reusable after thorough washing with PBS and sterilisation with 70% (v/v) ethanol / 30% (v/v) sterile water.

2.4. Cell analysis

Coomassie blue staining was employed to stain all the proteins within the cells, the stained whole cells can be used for quick analysis of cell density and morphology. Twenty bright field images of the stained cells were taken from 3 replicates obtained in independent experiments, cell densities (the percentage of the area covered by cells or cell colonies) from each image were then analysed using ImageJ (Rasband 1997). Cell viability was analysed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) staining. Briefly, cells on various substrates were washed thoroughly with PBS and incubated in MTT solution (0.5 mg/ml MTT in PBS) for 60 min at 37°C. MTT is converted in living cells by mitochondrial reductases into a purple formazan dye (Mosmann 1985), thus cells with high metabolic activity levels (viable cells) will be stained dark purple; the cells with low or no metabolic activities (less viable cells) will be stained either very light purple, or not at all. All the stained cells were then imaged and cell viability compared based on the signal intensity of each image. Fluorescence microscopy was also performed to identify astrocytes by labelling cell nuclei with 4',6-diamidino-2-phenylindole (DAPI, 300nM, Vector Laboratories Inc., Ca, USA), and GFAP with antibodies (anti-rabbit, Dako, Cambridgeshire, UK) as described previously (Sørensen et al. 2007). Student's unpaired t-test was used to compare cell densities in different culture conditions (the following indicators were used: * p<0.05, **p<0.01, ***p<0.001).

3. Results

3.1. Cell attachment on PCL substrates in perfusion cultures

Our preliminary experiments with Swiss rolls suggested that quick cell colonization within the scaffolds was crucial for perfusion culture. Thus our efforts were first focused on cell attachment on various substrates under perfused conditions. Astrocytes and fibroblasts were seeded separately on non-treated flat, pillared and grooved/pillared PCL substrates, incubated statically for 2 minutes, then perfused continuously at a fixed flow rate of 10 ml/hour (shear rate of $1.38 \times 10^{-2} \text{ s}^{-1}$) and monitored live using time-lapse microscopy. It was observed that almost all the astrocytes and fibroblasts quickly settled onto the substrates within 2 minutes' static incubation in the mini

chambers, as no cells were observed floating in the medium. However, the majority of the cells still remained rounded up instead of spreading onto the substrates. As perfusion started, a great proportion of these cells (approximately 40-50%) were picked up by the flowing medium, re-circulated in the perfusion system and thus did not have the chance to re-attach to the substrates, which was not influenced by the presence of either micro-pillars, micro-grooves or a combination of both as shown in Figure 2A. In a static control culture experiment, cells were seeded on NON-treated flat substrates incorporated into the mini bioreactor system, after having been incubated statically within the mini chamber for 45 minutes, which allowed the cells attach to the substrates. The cell loaded flat substrates were then removed from the chamber for subsequent static culture in 6-well tissue culture plates for up to 7 days. Although more cells were observed to spread on the substrates, a substantial number of cells remained rounded up after incubation for 2-6 hour, and even aggregated after static incubation for 5 to 7 days (Figure 2B). The cell loaded substrates were then incorporated into the bioreactor system for subsequent perfusion experiments. It was observed that all the rounded cells or cell aggregates were detached and re-suspended by continuous medium flow at 10ml/h for 10-20 minutes.

3.2. Evaluation of chemical features under perfused conditions

Preliminary static cell culture experiments indicated that both astrocytes and fibroblasts were able to quickly attach and spread onto P, P-PLL or P-COL treated PCL substrates within 5 to 10 minutes compared with NON, PLL or COL treated substrates where a substantial number of cells remained rounded up or aggregated even after static incubation for up to 7 days as shown in Figure 2B. Thus P-COL treated and non-treated flat PCL substrates were selected for further testing under perfused conditions. Astrocytes and fibroblasts were seeded separately on the substrates, incubated statically for 20, 60 and 120 minutes and then perfused continuously for 2 hours at flow rates of 0.3, 1.5, 5 and 20 ml/hour (equivalent shear rates: 4.16×10^{-4} , 2.08×10^{-4} , 6.94×10^{-4} and $2.77 \times 10^{-4} \text{ s}^{-1}$). As illustrated in Figure 3A and B, significantly higher densities of astrocytes (6-9%) and fibroblasts (26-29%)

were detected on P-COL treated substrates compared to the low cell densities (astrocytes 2-3%, fibroblasts 4-5%) on non-treated substrates after static incubation for 20min and perfusion for 2 hours, which was not obviously influenced by the different flow rates investigated. Almost identical results were obtained when the static incubation time was increased from 20 to either 60 or 120 minutes (data not shown), suggesting that most of the cells with the ability to firmly attach to the substrates could do so within 20 minutes.

3.3. Influences of medium perfusion on cell spreading and survival

Astrocytes and fibroblasts were seeded onto separate P-COL treated grooved/pillared substrates, statically incubated for 2 hours, and then perfused continuously overnight at a fixed flow rate of 10 ml/hour (equivalent shear rate: $1.38 \times 10^{-2} \text{ s}^{-1}$). Time lapse microscopy demonstrated that after having been continuously perfused for 18 hours, 40-50% of the attached astrocytes and fibroblasts remained rounded-up while only 10-40% did so in static culture carried out in separate 6-well tissue culture plates,, where most of the cells (60-90%) spread on the substrates as shown in Figure 4A. Further perfusion experiments demonstrated that almost all the cells were detached from the substrates by continuous perfusion for longer time periods (3 to 7 days).

In order to investigate how the adverse effect of continuous medium perfusion could be avoided, astrocytes and fibroblasts were seeded separately on Non-treated and P-COL treated substrates, incubated statically for 2 hours, then perfused continuously only for 10 minutes within every 2 hours with different flow rates of: 0.3, 1.5, 5 and 20 ml/hour (equivalent shear rates: 4.16×10^{-4} , 2.08×10^{-4} , 6.94×10^{-4} and $2.77 \times 10^{-4} \text{ s}^{-1}$). After non-continuously perfusion for 3 days, cell viability was analysed using MTT. As shown in Figure 4B and C, significantly more viable cells were found on all the P-COL treated PCL substrates (50-80%) than on non-treated substrates (1-6%).

3.4. Evaluation of the influences of microgrooves on cell alignments using perfusion cultures

Astrocytes and fibroblasts were seeded separately on P-COL treated grooved/pillared or pillared substrates, incubated statically in the mini bioreactor for 2 hours, then divided into 2 groups for static

in separate 6-well tissue culture plates or perfusion cultures in the mini bioreactor. For perfusion culture group, the cells were perfused every 2 hours for 10 minutes with flow rates of: 0.3, 1.5, 5 and 20 ml/hour (equivalent shear rates: 4.16×10^{-4} , 2.08×10^{-4} , 6.94×10^{-4} and $2.77 \times 10^{-4} \text{ s}^{-1}$). After 3 days of either static culture in separate 6-well tissue culture plates, or perfusion culture in the mini bioreactor, cell morphology and distribution on the substrates were analysed. It was found that the micro-grooves maintained their influences on cell alignment in perfusion culture, as cells were observed to align along the grooves and ridges on the grooved substrates, and remain randomly oriented on pillared substrates as shown in Figure 5A. Further image analysis indicated that aligned astrocytes were almost equally distributed on either ridges or within the grooves and not obviously influenced by medium perfusion (Figure 6A). However, medium perfusion influenced fibroblast positioning significantly, as in static culture fibroblasts aligned to the grooves and were equally distributed on both ridges and within grooves, whereas in perfusion culture most of the cells (80-90%) were found within grooves, as shown in Figures 5B and 6B.

4. Discussion

During the past decade, considerable research efforts have been focused on the fabrication of 3D scaffolds for tissue engineering (Hubbell, 2003; Stevens and George, 2005; Zong, 2005) based on the observations of cells on various substrates in static cultures (Britland *et al.*, 1996; Wojciak-Stothard *et al.*, 1996; Flemming *et al.*, 1999; Curtis and Wilkinson, 2001; Anderson, 2003, Stevens and George, 2005). Since some of the engineered scaffolds are likely to be subject to perfusion culture (Jelinek *et al.*, 2000; Salem *et al.*, 2002; Link *et al.*, 2004; Sun *et al.*, 2005), it is essential to test their biological performances not only under static but also under perfused culture conditions. A preliminary effort to perfuse cells including astrocytes and fibroblasts loaded into our previously developed tubular constructs ('Swiss-rolls') demonstrated that medium perfusion adversely influenced cell adhesion and survival, which was difficult to address due to the complex 3D structure

and large size of the tubular constructs. Thus the aim of this research was to develop a mini-bioreactor system, which could be used to investigate the influences of chemical and topographical cues on cell adhesion and survival under perfused conditions. The bioreactor system displayed several technical and operational advantages. First of all, it was easy to fabricate and operate. Secondly, it was possible to directly observe the cells during perfusion on various PCL substrates as it could be integrated with time-lapse microscopy. Thirdly, multiple, parallel perfusion chambers facilitated the running of simultaneous comparative experiments under different conditions. Finally, apart from PCL substrates, any other substrate transparent or not, can be evaluated using this system. In this research, the bioreactor system presented a challenge in tissue culture using 3D scaffolds under perfused conditions. Due to the very small volumes within the scaffold and normally high cell density, cells will need effective and efficient medium perfusion to support cell survival. Thus quick and firm cell adhesion to withstand medium perfusion is of prime importance to allow medium perfusion in 3D tissue cultures to start early, as static culture processes might not always be applicable. For example, the culture of astrocytes on PLL treated (Sørensen *et al.* 2007) or non-treated PCL substrates is a normal practice in static culture as the cells are exposed to the bulk medium and have sufficient time (days or even weeks) to attach to the substrates. Although a substantial number of cells remained rounded up or aggregated on these substrates even after static incubation for up to 7 days, this practice is also applicable for static culture due to relatively high cell seeding density, sufficient medium and culture periods for cell to attach. However, this is not applicable to perfusion culture mainly due the limited space/medium within the 3D scaffold and short static culture time period for firm cell attachment before perfusion is initiated for medium supply and waste removal, which echoed our preliminary perfusion of the cell-loaded ‘Swiss-rolls’. Investigation of substrates under perfused conditions indicated that both astrocytes and fibroblasts were able to attach to the optimised P-COL treated substrates within 20 minutes and resist immediate continuous medium perfusion for at least 2 hours. Although medium perfusion was

supposed to enhance nutrient supply and waste removal within 3D scaffolds, our research clearly demonstrated its adverse influences as continuous perfusion was shown to interfere with cell spreading and even cell survival. Fortunately, these adverse influences of continuous medium perfusion could be reduced or balanced by pulse perfusion.

Microgrooves still maintained their influences on cell orientation and alignment under perfused conditions as established using the bioreactor system. Moreover, medium perfusion also demonstrated subtle cell type specific influences on cell behaviours, as 80-90% of fibroblasts were confined within the grooves in perfusion culture, whilst in static culture controls they were observed to distribute almost equally on ridges and in grooves. Astrocytes, the other cell type studied, showed no such differential behaviour, which could probably be due to stronger cell-cell interactions, as astrocytes tend to form tight colonies, thus cell migration was not as easily influenced by flowing medium as compared to the fibroblasts which remained individuals. This observation suggested that the fine control of medium perfusion (shear rate, intermittent flow etc.) could be integrated with other strategies such as chemical surface modifications, and topography to engineer and manipulate cellular behaviours such as cell growth and/or alignment differentially for various applications.

Overall, a gap exists in our current knowledge of cell behaviours on structured substrates and its applications to tissue engineering within 3D scaffolds as these are normally designed based on the results obtained with static cell cultures on sutured substrates, while some of them are used for perfusion cultures. The aim of this research was to develop a scale down bioreactor system, which would allow the optimisation of various chemical and topographical cues in our previously developed tubular ϵ -polycaprolactone scaffold under perfused conditions. The investigation of the behaviour of two exemplary cell types (fibroblasts and cortical astrocytes) using the miniaturized bioreactor indicated that: (1) quick and firm cell adhesion was critical for perfusion culture compared with static culture, and that it was necessary to evaluate cell attachment under perfused conditions, (2) continuous medium perfusion adversely influenced cell spreading and survival,

which could be balanced by pulse perfusion, (3) micro-grooves still maintained their influences on cell alignment under perfused conditions, while medium perfusion demonstrated an additional influence on fibroblast positioning, but not on astrocyte positioning on the grooved substrates. This research demonstrated that the development of the mini-bioreactor system represents a significant advance in the design and fabrication of various 3D scaffolds by allowing one to bridge the gap between well documented observations about the influences of different chemical and topographical cues on cellular behaviors in static culture and their actual application within large scale perfusion culture for the generation of engineered 3D tissues.

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FIGURE LEGENDS

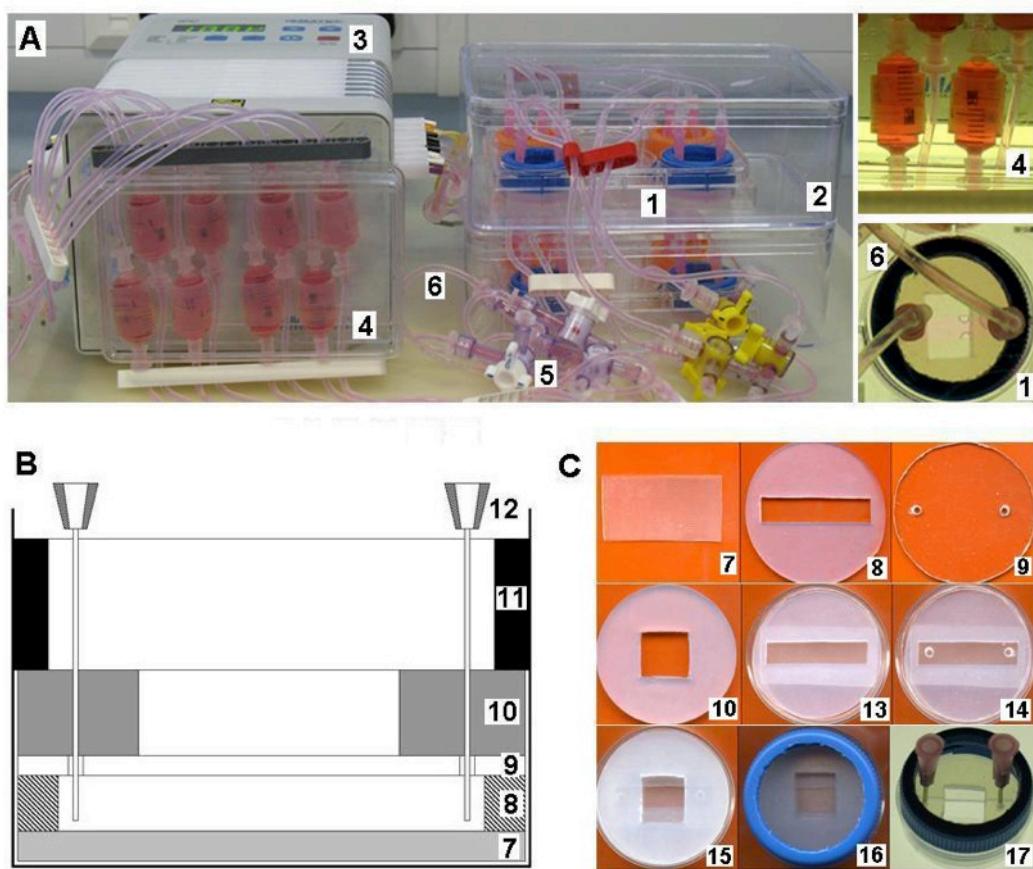


Figure 1. (A) Appearance of the bioreactor system with multiple mini perfusion chambers. (B) Schematic diagram and (C) components and appearance of the mini perfusion chamber. The bioreactor was consisted of two commercially available 6-well tissue culture plates (1) situated in two plastic boxes (2), an 8-channel peristaltic pump (3), 8 medium reservoirs (4) and 3-way valves (5), all of which were connected with silicone tubing (6). Eight mini perfusion chambers were constructed in the two 6-well plates. A sandwich of the following parts was assembled inside the wells to form the perfusion chambers: a piece of rectangular PCL substrate (7), a circular silicone spacer (8) with a rectangle chamber created inside, a circular transparent polystyrene plate (9) with two small holes that were 20mm apart to each other, a circular silicone sealing plate (10) with an observation window, a plastic o-ring (11) and two needles (12). The PCL substrate and the rectangular chamber inside the silicone spacer formed the mini perfusion chamber (13), which was sealed by the polystyrene plate (14). The two holes in the polystyrene plate were sealed by the thick silicone plate (15), which was further clamped by the plastic o-ring (16). The two needles inserted into the perfusion chamber through the thick silicone sealing plate and the holes on the polystyrene plate were used as the inlet and outlet of the perfusion chamber respectively (17).

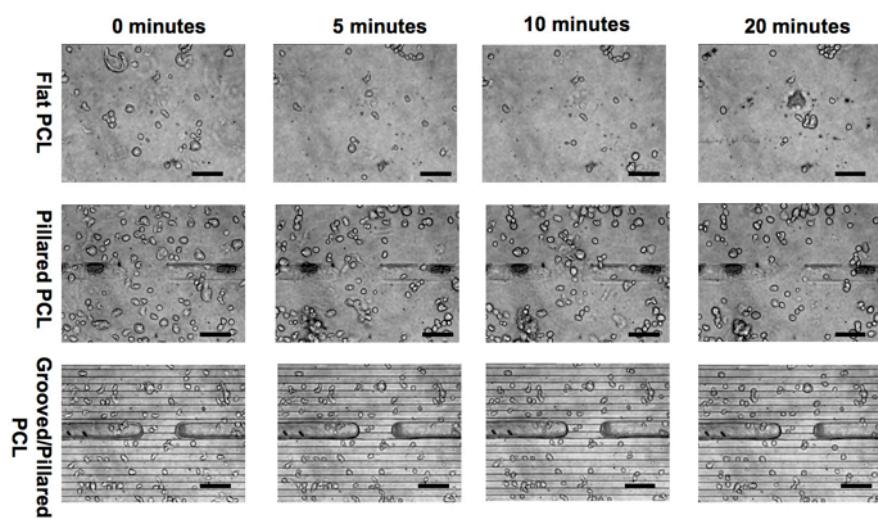
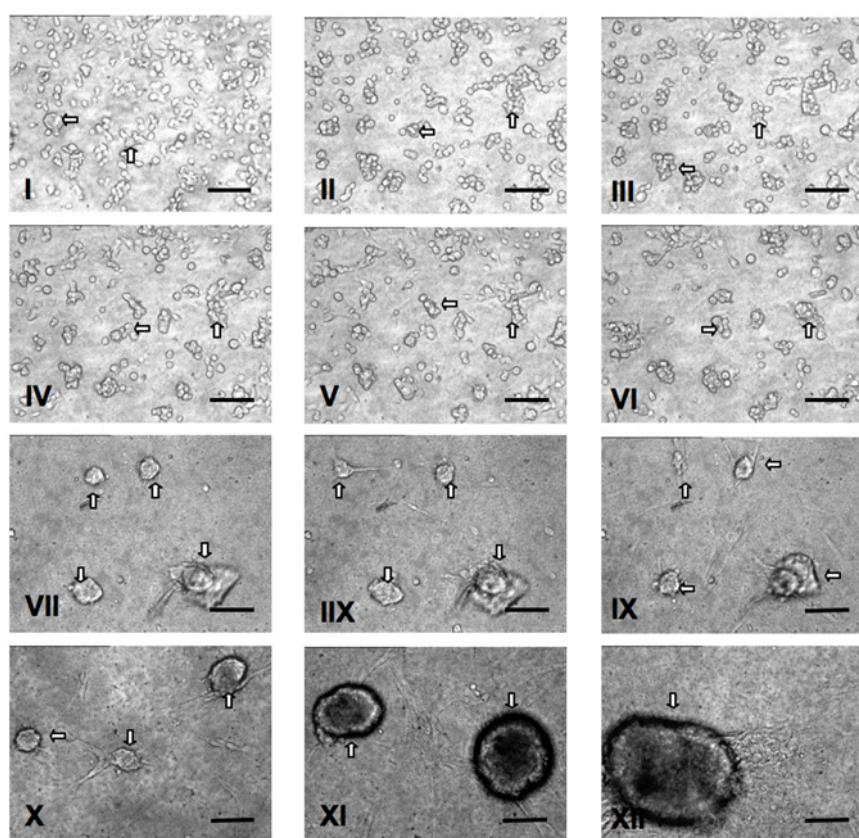
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Figure 2. (A) Micrographs from time-lapse videos of astrocytes seeded on non-treated flat, pillared, or grooved/pillared polycaprolactone (PCL) substrates fabricated in the bioreactor system with

multiple mini perfusion chambers, incubated statically for 2 minutes, then perfused continuously with medium at a defined flow rate of 10ml/hour and videoed for 0, 5, 10 and 20 minutes.

(B) Micrographs from time-lapse videos of astrocytes seeded on non-treated flat polycaprolactone (PCL) substrates, incubated for 2 minutes and then videoed at 0 minute (I), 5 minutes (II), 10 minutes (III), 20 minutes (IV), 30 minutes (V) and 4 hour (VI) and of fibroblasts cultured on non-treated flat PCL substrates and then videoed at day 2 (VII), day 3 (VIII), day 4 (IX), day 5 (X), day 6 (XI) and day 7 (XII). The round-up cells and/or cell aggregates are pointed with arrows. Bar = 100 μ m.

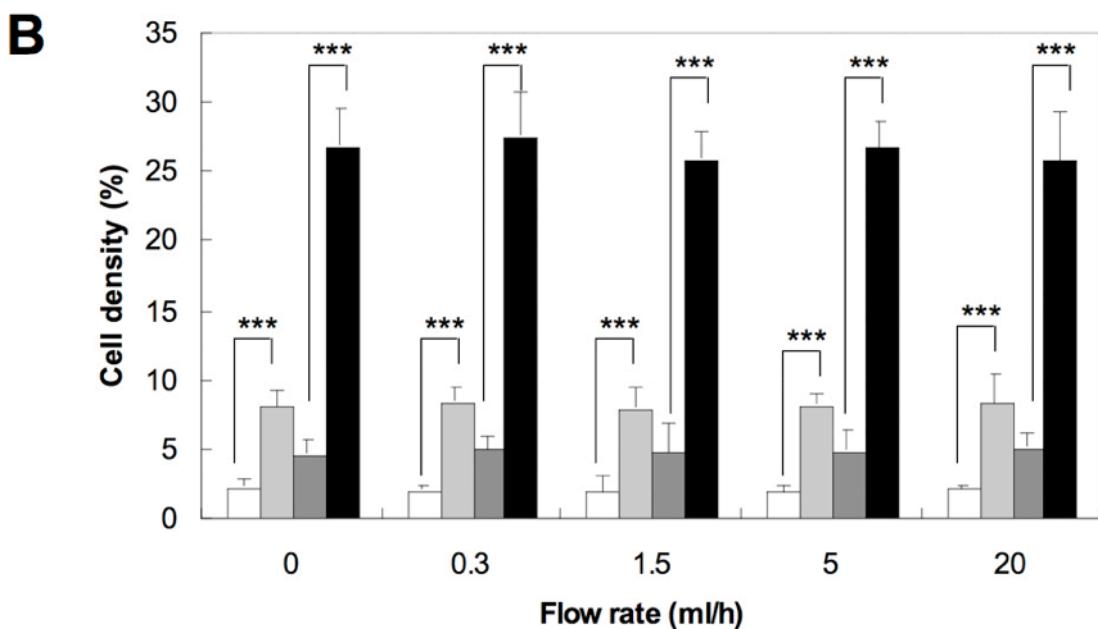
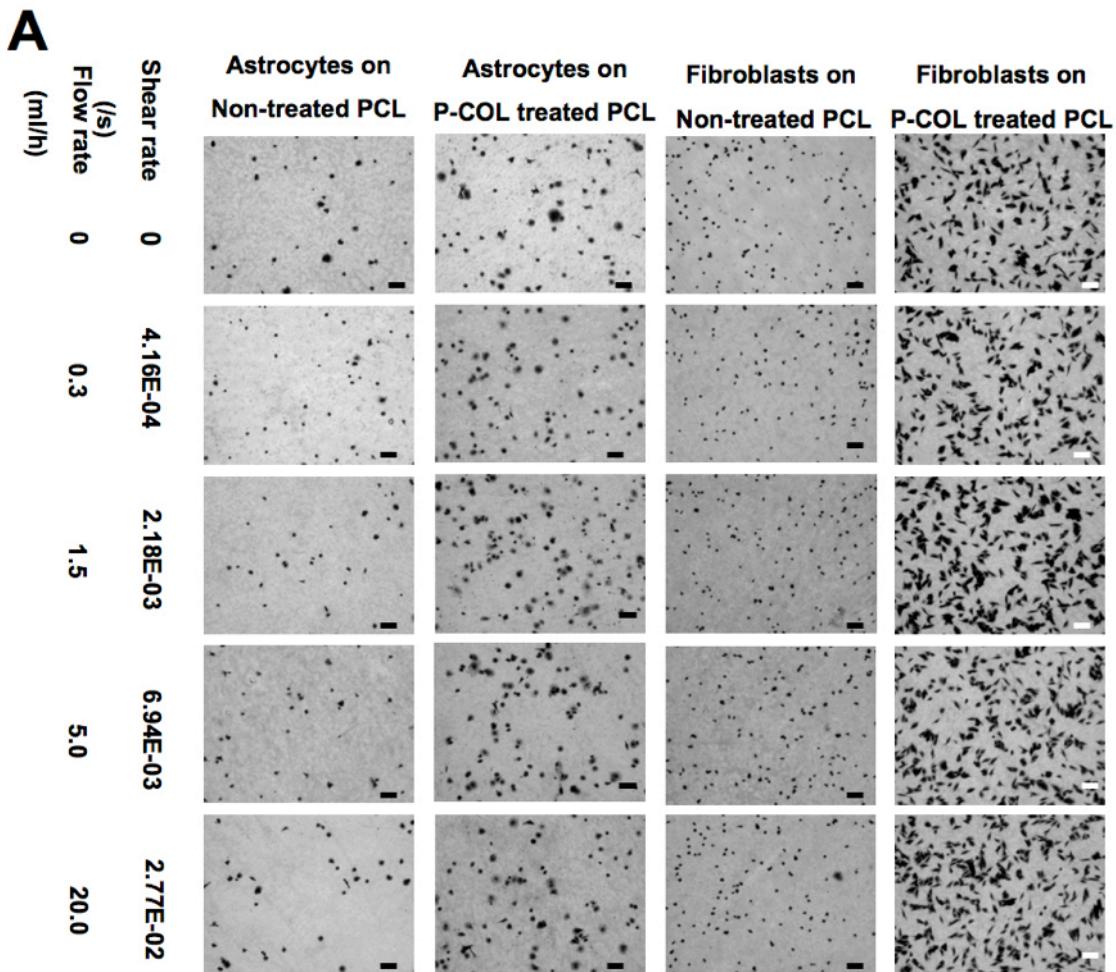


Figure 3. (A) Micrographs of astrocytes and fibroblasts seeded on non-treated or P-COL treated flat polycaprolactone (PCL) substrates fabricated in the mini perfusion chambers of the bioreactor

system, incubated statically for 20 minutes, perfused continuously for 2 hours with medium at varying flow rates (0 ml/hour or static culture control, 0.3, 1.5, 5 and 20 ml/hour) or shear rates (0, 4.16×10^{-4} , 2.08×10^{-4} , 6.94×10^{-4} and $2.77 \times 10^{-4} \text{ s}^{-1}$), then stained with Coomassie Blue and imaged. (B) Cell densities of astrocytes on NON-treated (white column) and P-COL treated (light grey column) substrates and fibroblasts on NON-treated (dark grey column) and P-COL treated (black column) substrates were analysed using image J and plotted. The treatment methods used were: (P-COL): treated with plasma for 5 minutes then coated with collagen. (NON): non-treated PCL substrates. Results shown are mean \pm SD (n=3), Bar = 100 μm .

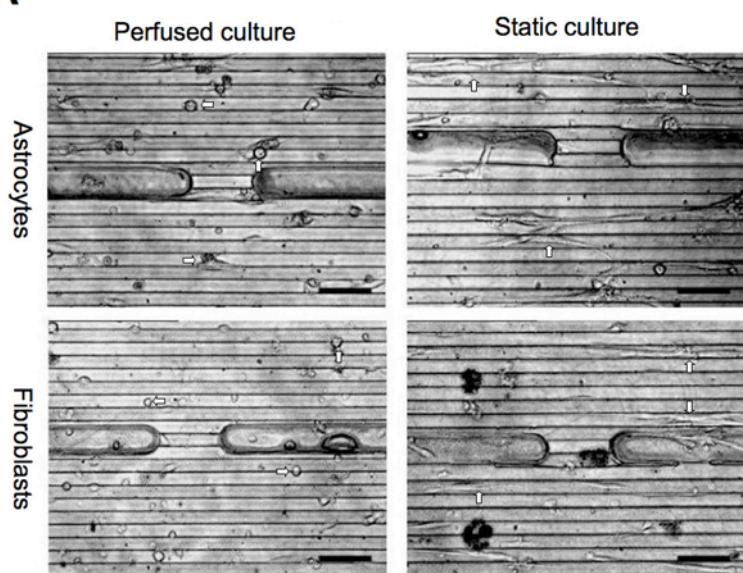
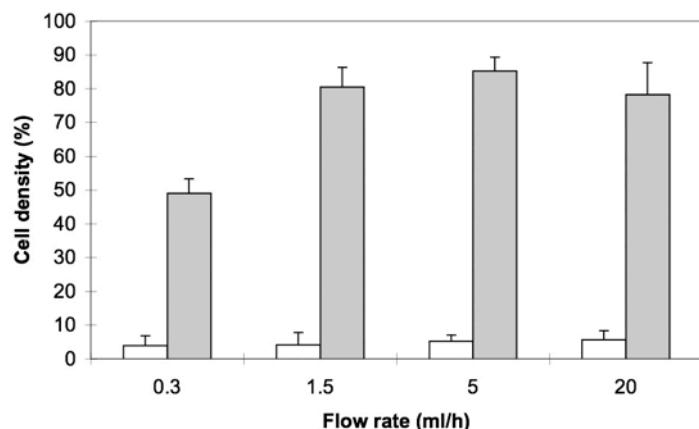
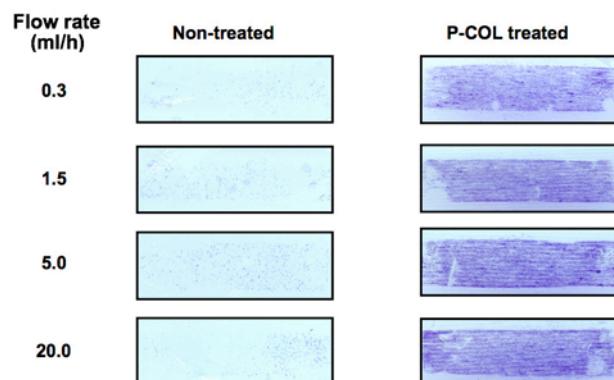
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Figure 4. (A) Micrographs from time-lapse videos of astrocytes and fibroblasts cultured statically or perfused continuously at a defined flow rate of 10ml/hour for 18 hours on non-treated

grooved/pillared PCL substrates fabricated in the bioreactor system. The round-up cells in perfusion cultures and spread cells in static cultures are pointed with arrows. Bar = 100 μ m. (B) Cell densities of viable astrocytes on NON-treated (white column) and P-COL treated (light grey column) substrates after incubated statically for 2 hours, perfused continuously for 10 minutes within every 2 hours with medium at varying flow rates (0.3, 1.5, 5 and 20 ml/hour) or shear rates (4.16×10^{-4} , 2.08×10^{-4} , 6.94×10^{-4} and 2.77×10^{-4} s⁻¹) for 3 days, stained with MTT, analysed using image J and plotted. Results shown are mean \pm SD (n=3), Bar = 100 μ m. (C) The appearances of MTT stained astrocytes seeded on non-treated and P-COL treated polycaprolactone (PCL) substrates fabricated in the mini perfusion chambers, incubated statically for 2 hours, perfused continuously for 10 minutes within every 2 hours with medium at varying flow rates (0.3, 1.5, 5 and 20 ml/hour) for 3 days.

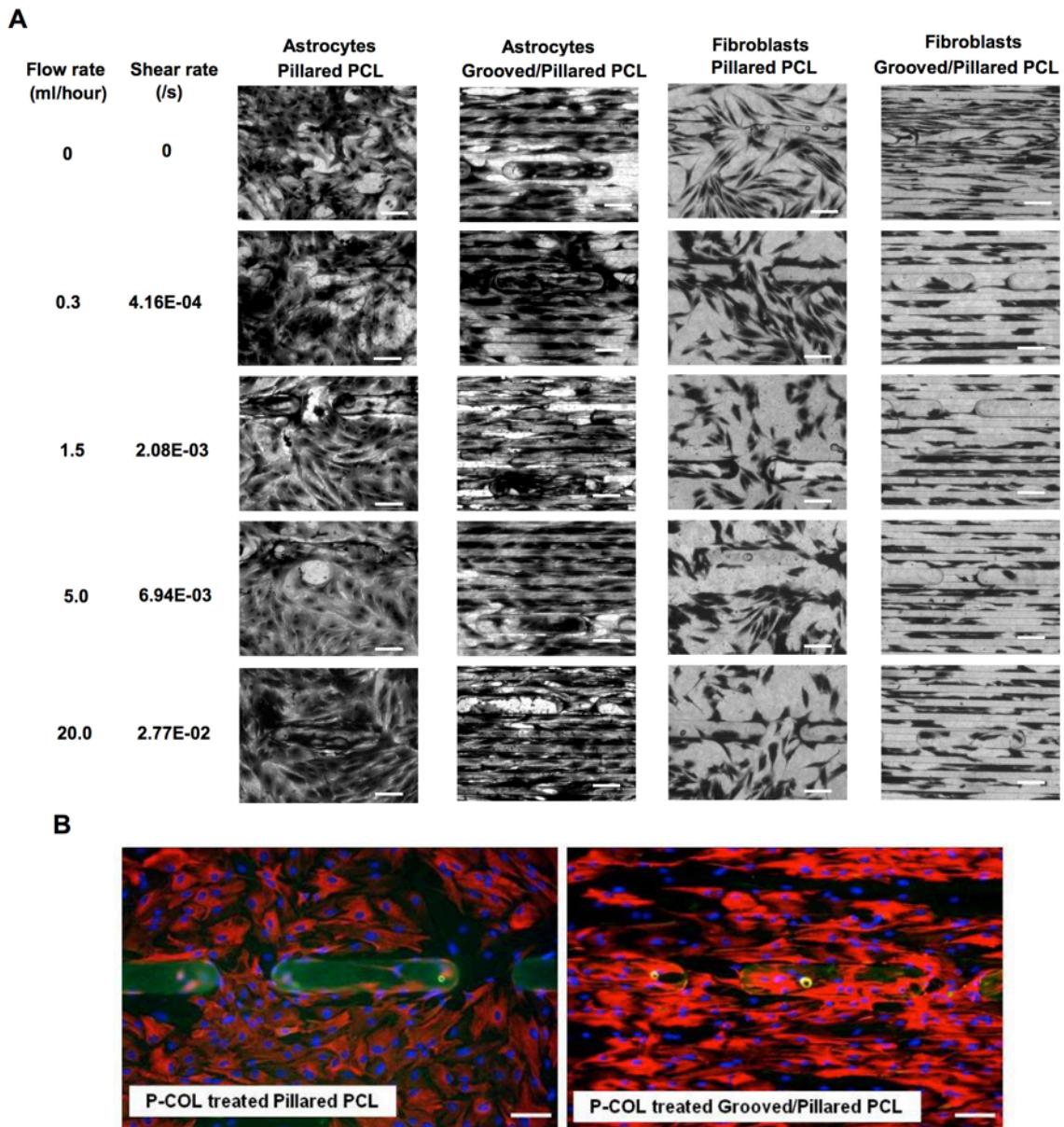


Figure 5. (A) Micrographs of Coomassie Blue stained astrocytes and fibroblasts seeded on P-COL treated pillared and grooved/pillared polycaprolactone (PCL) substrates fabricated in the mini perfusion chambers, incubated statically for 2 hours, perfused continuously for 10 minutes within every 2 hours with medium at varying flow rates (0 ml/hour or static culture control,, 0.3, 1.5, 5 and 20 ml/hour) or shear rates ($0, 4.16 \times 10^{-4}, 2.08 \times 10^{-4}, 6.94 \times 10^{-4}$ and $2.77 \times 10^{-4} \text{ s}^{-1}$) for 3 days. Bar = 100 μm . (B) Epifluorescence micrographs of the immuno-stained astrocytes on P-COL treated pillared and grooved/pillared PCL substrates after perfused intermittently with the fixed flow rate of

5ml/h for 3 days, the cells were stained with DAPI (Blue) and GFAP (Red). The treatment methods used were: (P-COL): treated with plasma for 5 minutes then coated with collagen. Bar = 100 μ m.

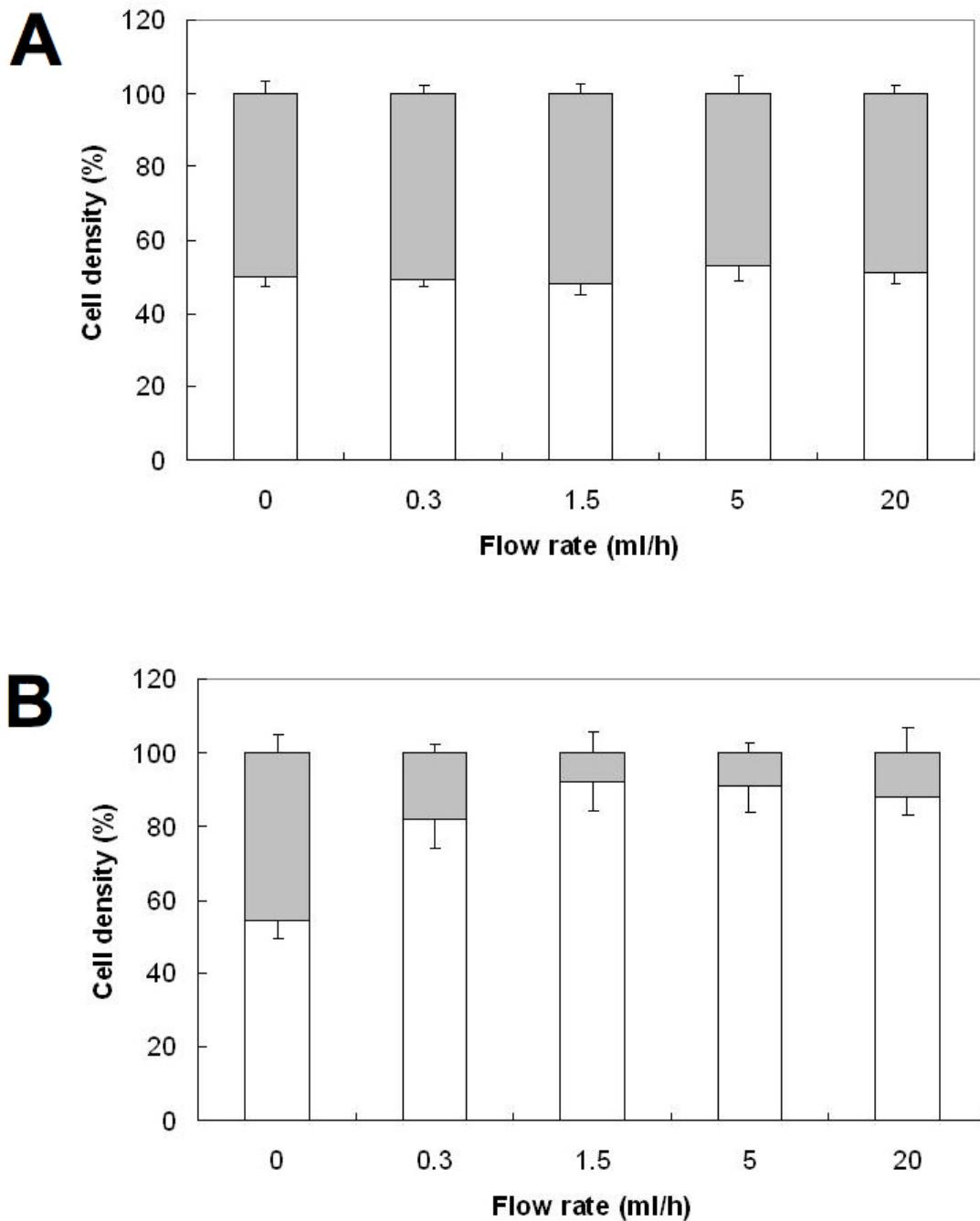


Figure 6. Distribution of (A) astrocytes and (B) fibroblasts in the grooves (white column) and on the ridges (light grey column) of the micro-grooved substrates after perfused intermittently with varying

flow rates (0 ml/hour or static culture control, 0.3, 1.5, 5 and 20 ml/hour) or shear rates (0 , 4.16×10^{-4} , 2.08×10^{-4} , 6.94×10^{-4} and $2.77 \times 10^{-4} \text{ s}^{-1}$) for 3 days. Results shown are mean \pm SD ($n=3$),