

**A PREDICTION OF CELL DIFFERENTIATION AND PROLIFERATION
WITHIN A COLLAGEN-GLYCOSAMINOGLYCAN SCAFFOLD
SUBJECTED TO MECHANICAL STRAIN AND PERFUSIVE FLUID FLOW**

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ABSTRACT

Background: Mesenchymal stem cell (MSC) differentiation can be influenced by biophysical stimuli imparted by the host scaffold. Yet, causal relationships linking scaffold strain magnitudes and inlet fluid velocities to specific cell responses are thus far underdeveloped. This investigation attempted to simulate cell responses in a Collagen-glycosaminoglycan (CG) scaffold within a bioreactor. **Methodology:** CG scaffold deformation was simulated using μ -Computed Tomography (CT) and an in-house finite element solver (FEEBE/*linear*). Similarly, the internal fluid velocities were simulated using the aforementioned μ CT dataset with a computational fluid dynamics solver (ANSYS/CFX). From the ensuing cell-level mechanics, albeit octahedral shear strain or fluid velocity, the proliferation and differentiation of the representative cells were predicted from deterministic functions. **Results:** Cell proliferation patterns concurred with previous experiments. MSC differentiation was dependent on the level of CG scaffold strain and the inlet fluid velocity. Furthermore, MSC differentiation patterns indicated that specific combinations of scaffold strains and inlet fluid flows causes phenotype assemblies dominated by single cell types. **Discussion:** Further to typical laboratory procedures, this predictive methodology demonstrated loading-specific differentiation lineages and proliferation patterns. It is hoped these results will enhance *in-vitro* tissue engineering procedures by providing a platform from which the scaffold loading applications can be tailored to suit the desired tissue.

KEYWORDS: Collagen-Glycosaminoglycan scaffold, Perfusion Bioreactor, Tissue Engineering.

1 INTRODUCTION

Tissue engineering requires cells to differentiate and proliferate within artificial scaffolds. These scaffolds provide structural support and nutrient transport to cells, which in turn allow cell functions to be tailored to suit the desired tissue [McMahon *et al.*, 2008]. By externally straining or imposing fluid to flow through, complex micro-environments of deformation transpire. In fact, due to their architectural heterogeneity, many porous materials endure compounding fluid-solid interactions that make measuring such micro-environments exceedingly difficult [Holmes and Mow, 1990; Gu *et al.*, 2004]. It is however, these micromechanical deformations that impart biophysical stimuli onto the cells, and so it is these micro-environments that hold the key to understanding how cells function within artificial scaffolds.

Mechanoregulatory models can predict cell function and tissue growth. Accordingly, biophysical stimuli regulates skeletal tissue growth, albeit in bone fracture healing [Carter and Beaupré 2001; Lacroix and Prendergast, 2002], distraction osteogenesis [Isaksson *et al.*, 2006], osteochondral defect healing [Kelly and Prendergast, 2005], bone ingrowth into porous implants [Andreykiv *et al.*, 2005], *in-vitro* growth of articular cartilage [Ficklin *et al.*, 2009] and tissue engineering [Byrne *et al.*, 2007]. Interestingly, mechanoregulatory models that combine octahedral shear strain and interstitial fluid velocity as tissue growth determinants prove most accurate [Isaksson *et al.*, 2006]. In particular, by comparing a finite element (FE) simulation to distraction osteogenesis within an ovine bone specimen, Isaksson *et al.* (2006) showed tissue formation can be accurately predicted using octahedral shear strain and fluid velocity. Furthermore, McMahon *et al.* (2008) recently confirmed strain and fluid velocities to be the most influential stimuli on cell behaviour in a CG scaffold.

CG scaffolds display many positive indicators for tissue engineering, including cell mechanics [Harley *et al.*, 2008; Schulz Torres *et al.*, 2000], cell growth [Farrell *et al.*, 2006; McMahon *et al.*,

2008], mechanical loading [Harley *et al.*, 2007; Haugh *et al.*, 2009] and fluid flow within bioreactors [Jungreuthmayer *et al.*, 2008]. This paper intends to explore the biophysical stimuli within a CG scaffold to further enhance this promising construct. Importantly, a timeframe spanning the initial 14 days after seeding, which is prior to matrix deposition and microstructural changes [Farrell *et al.*, 2006], will be investigated. Specifically, this paper intends to,

1. Quantify cell numbers and cell differentiation pathways resulting from local strains and interstitial fluid velocities within a CG scaffold (arising from the biophysical loading imparted by a typical bioreactor).
2. Determine the magnitudes of apparent strains and inlet fluid velocities, applied by a typical bioreactor, that encourages the growth of bone, cartilage and fibrous tissue.
3. Compare the findings of this predictive study with published data to assess model validity.

2 METHODOLOGY

In the following two sections, methodologies are presented to 1) describe how the architecture of a CG scaffold was imaged and then subsequently used to generate FE and CFD models, respectively, and then 2) how cell functions were represented using descriptions from published experiments.

2.1 CG Scaffold Representation

Image Thresholding Procedure

Given that the microstructure between CG multiple scaffold samples varies by only 0.5% [O'Brien *et al.*, 2004], only one CG scaffold, manufactured using a lyophilisation process at a freezing drying temperature of -40°C and dehydrothermal treatment, crosslinked at 105°C for 24 hours in a large stainless steel pan of 169x253mm [O'Brien *et al.*, 2004], was characterised using μ CT-imaging (6 μ m) [SCANCO, Switzerland]. With purpose-written thresholder (MATLAB[®]) and mesher (Intel[®] FORTRAN90) codes, a 3D interconnecting microstructure that characterised the entire depth of the

CG scaffold (1.5 x 1.5 x 3.9mm) was generated. To ensure mesh accuracy, the microstructural stress magnitudes within the mesh were corroborated by a supplementary sub-micron resolution mesh (SKYSCAN, Belgium).

Scaffold straining (tension or compression) can provide a means to initiate cell differentiation [Ignatius *et al.*, 2005; McMahon *et al.*, 2008; Thorpe *et al.*, 2008], while fluid flow through the scaffold can encourage cell proliferation [Cartmell *et al.*, 2003; Jassma and O'Brien, 2008; Schulz *et al.*, 2008]. Thus, to include both forms of biophysical stimulus (scaffold strain and fluid flow), the bioreactor design by Schulz *et al.* (2008) was simulated (Figure 1a).

FE Model Formulation

FEEBE/linear, an in-house Intel® FORTRAN90 small-strain FE element-by-element solver (checked and verified in Harrison *et al.*, 2008), simulated uniaxial compression of the CG scaffold (FE mesh had 6.4 million voxel elements). Small strain kinematics were assumed because, 1) the majority of microscopic strains within highly-porous scaffolds are the same order of magnitude as the applied scaffold strain [Lacroix *et al.*, 2006; Sandino *et al.*, 2008], 2) the apparent strains in this investigation are within the elastic region (<5%), and 3) initial investigations showed that it would be computationally impractical to attempt a non-linear kinematics solution with 6.4million elements. With a constant elastic modulus of 16.32kPa (no matrix changes were expected within the 14 day timeframe [Farrell *et al.*, 2006]) and symmetric boundary conditions that enforced a periodic unit-cell [Böhm, 2004], 1, 2 and 5% compressive strains were applied to the modelled scaffold (Figure 1b).

CFD Model Formulation

A fluid mesh, generated from the non-solid phase of the FE mesh, was solved by ANSYS® CFX® (CFD mesh had 40.1 million voxel elements). Importantly, for a $235\mu\text{ms}^{-1}$ inlet fluid velocity (flow

rate $Q=1$ ml/min), the relative deformation, or the relative structural difference between an undeformed and deformed CG scaffold, caused by interstitial fluid flow is less than 0.2% [Jungreuthmayer *et al.*, 2009]. Such minimal structural deformation from fluid flow can be attributed to two factors: CG scaffold permeability is relatively high ($\sim 10^{-10}$) [Tierney *et al.*, 2009; O'Brien *et al.*, 2007] and the inlet fluid velocity of $235\mu\text{ms}^{-1}$ is relatively low: meaning negligible fluid drag forces and negligible solid compaction. Thus, the walls of the scaffold can be considered rigid for an inlet fluid velocity below $235\mu\text{ms}^{-1}$. Consequently, as previously implemented [Sandino *et al.*, 2008], an uncoupled fluid analysis simulated a perfusion bioreactor [Schulz *et al.*, 2008]. The bioreactor was not expected to have fluid flow around and into the sides of the scaffold as an O-ring prevented peripheral flow [Jaasma *et al.*, 2008] (Figure 1a). Consequently, with symmetric boundary conditions applied to the four external sides (planes of flow and geometry), an inlet surface at the air-side and a zero pressure outlet at the pan-side was enforced. With laminar flow, incompressible Newtonian fluid with a viscosity of 0.001Pas and no-slip scaffold walls, a range of 1, 10 and $100\mu\text{ms}^{-1}$ inlet velocities were implemented. (Figure 1c).

2.2 Cell Representation

Cell Description

As cells exhibit an elastic modulus some 3 orders of magnitude lower than the modulus of a CG scaffold strut (1kPa [Thoumine *et al.*, 1999] versus 5.28MPa [Harley *et al.*, 2007]), cells were considered to strain according to microstructural scaffold deformation. Consequently, cells were represented as 2D virtual areas with no stiffness, where the triangular line segments corresponded to cell lengths of $\sim 50\mu\text{m}$ [Zaleskas *et al.*, 2004] (Figure 2a). From standard FE equations that govern 3-noded membrane deformation, in-plane octahedral shear strains γ were computed for each virtual cell from the scaffold nodal displacements calculated in the FE simulation (Figure 2a). From the CFD analyses, cell-specific fluid velocities v were calculated from the mean interstitial velocity (velocity magnitude) within a volume ($60 \times 60 \times 60\mu\text{m}$) surrounding the cell (Figure 2b); there were

no cells in the CFD meshes so the volume of space occupied by the cell determined cell-specific fluid velocity.

Cell Formulae

Using a purpose-written Intel[®] FORTRAN90 code, a cell mechanoregulatory algorithm evaluated the FE and CFD results. The algorithm iterated over 10 million cycles, whereby one cell was randomly selected per iteration. For each iteration, the response of the chosen cell was determined depending on the biophysical stimulus (Figure 3). The mechanoregulatory algorithm predicted the fate of MSC cells and specialised cells (SPC), which included osteoblast (OST), chondrocyte (CHD) and fibroblast (FBT) cells. Differentiation applied only to MSC cells. MSC cells were considered to divide symmetrically into two SPC cells, albeit an OST, CHD or FBT, such that,

$$\text{MSC} \rightarrow 2\text{SPC} \quad (1)$$

An essential age (iteration>7 [Byrne *et al.* 2007]) before MSC differentiated simulated the role of progenitor cells as an intermediary between MSC and SPC cells. Likewise, a critical age was implemented for MSC death (iteration>20) to replicate the typical longevity of a cell. Phenotype selection was based on the mechanoregulatory relationship proposed by Prendergast *et al.* (1997), where cell phenotype was dependent on the biophysical stimuli S ,

$$S = \frac{\gamma}{3.75 \times 10^{-2}} + \frac{v}{3 \times 10^{-6}} \quad (2)$$

$$\gamma = \sqrt{\frac{2}{3}} \sqrt{\varepsilon_1^2 - \varepsilon_1 \varepsilon_2 + \varepsilon_2^2} \quad (3)$$

S was a function of γ , the in-plane octahedral shear strain experienced by the cell, and v , the interstitial fluid velocity surrounding the particular cell. Note that the 2D virtual cell generates a state of 2D in-plane strain, thus only ε_{11} , ε_{22} and ε_{12} are non-zero and equation (3) implements just the in-plane principal strains ε_1 and ε_2 . The phenotype became FBT when $S > 3$, CHD when $1 <$

$S < 3$ and OST when $S < 1$ [Perez and Prendergast, 2007; Byrne *et al.*, 2007].

A cell proliferation potential P , where subscripts denote phenotype, included the net result of proliferation minus necrosis and was defined as,

$$\begin{bmatrix} p_{MSC} \\ p_{FBT} \\ p_{CHD} \\ p_{OST} \end{bmatrix} = \begin{bmatrix} a_{MSC} & 0 & 0 & 0 & 0 & 0 \\ a_{FBT} & b_{FBT} & c_{FBT} & d_{FBT} & e_{FBT} & f_{FBT} \\ a_{CHD} & b_{CHD} & c_{CHD} & d_{CHD} & e_{CHD} & f_{CHD} \\ a_{OST} & b_{OST} & c_{OST} & d_{OST} & e_{OST} & f_{OST} \end{bmatrix} \begin{bmatrix} 1 \\ v \\ v^2 \\ 1 \\ \gamma \\ \gamma^2 \end{bmatrix} \quad (4)$$

The constant a_{MSC} was defined as 0.3 [Perez and Prendergast, 2007], while the remaining constants were calculated from published experimental data that coincided with the 14 day post-seeding timeframe of this investigation (explained below).

A perfusion bioreactor [Cartmell *et al.*, 2003] provided data on cell proliferations for scaffold inlet flow rates Q , of 0.01, 0.1, 0.2 and 1 ml/min. The inlet flow rates were converted to cell-level fluid velocities by an analytical method [Jungreuthmayer *et al.*, 2008], where the mean interstitial velocities within the scaffold, u_{scaff} , are,

$$u_{scaff} = \frac{Q}{\pi \Phi r^2} \quad (5)$$

where Φ is the scaffold porosity and r is the chamber radius [Cartmell *et al.*, 2003]. Note equation (5) estimates *interstitial* velocities within the scaffold (u_{scaff}) from *external* inlet velocities applied to the scaffold (Q). A quadratic function ($R^2 = 1$) was then used to describe the relationship between cell proliferation (P) and mean interstitial scaffold velocity (u_{scaff}), such that

$$P_{\alpha} = -8 \times 10^{-5} u_{scaff}^2 - 0.0041 u_{scaff} + 1.3205 \quad \alpha = \text{FBT, CHD, OST} \quad (6)$$

The constants of equation (6) relate to the constants a , b and c of equation (4): a_{α} equals 1.3205, b_{α} - 0.0041 and c_{α} - 8×10^{-5} .

An *in-vitro* cell strain method [Song *et al.*, 2007] provided cell proliferation values as a function of applied scaffold strain ($\varepsilon = 0.02, 0.04$ and 0.08). By converting the applied strains into octahedral shear strains γ , a quadratic function ($R^2 = 0.9902$) described the relationship between cell proliferation (P) and octahedral shear strain γ , such that

$$P_{\alpha} = -47.699 \gamma^2 + 8.3115 \gamma + 0.0077 \quad \alpha = \text{FBT, CHD, OST} \quad (7)$$

The constants of equation (7) relate to the constants d , e and f of equation (4): d_{α} equals 0.0077, e_{α} 8.3115 and f_{α} 0.0077.

In a novel extension of the Prendergast *et al.* (1997) model, outer limits of biophysical stimuli were used to signify cell necrosis. Thus, a maximum interstitial velocity of $105.4 \mu\text{m s}^{-1}$ [Cartmell *et al.*, 2003] and a maximum octahedral shear strain of 0.175 [Song *et al.*, 2007] resulted in,

$$S2 = \frac{v}{105.4 \times 10^{-6}} + \frac{\gamma}{0.175} \quad (8)$$

$S2$ represents a critical limit where a value above 1 denotes necrosis (Figure 3). In summary, the constants of equation (4) equal $a_{MSC} = 0.3$; $a_{FBT} = a_{CHD} = a_{OST} = 1.3205$; $b_{FBT} = b_{CHD} = b_{OST} = -0.0041$; $c_{FBT} = c_{CHD} = c_{OST} = -8 \times 10^{-5}$; $d_{FBT} = d_{CHD} = d_{OST} = 0.0077$; $e_{FBT} = e_{CHD} = e_{OST} = 8.3115$; $f_{FBT} = f_{CHD} = f_{OST} = -47.699$.

Given the direction and speed of cell migration is influenced by the surrounding extracellular matrix, a probability indicator dictated that 69% [Harley *et al.*, 2008] of the seeded cells were

motile at any given time.

Finally, using a 3D lattice (25 x 25 x 65) of the cell compartments (Figure 2), cell positions were monitored throughout the analyses. Within each compartment, one cell could occupy the space (if chosen by the mechanoregulatory algorithm) which resulted in a maximum cell density of 4.63×10^6 cells/cm³. Additionally, the local cell density also contributed to the proliferation potential. Specifically, the local cell density was calculated by monitoring the neighbouring environments, whereby the neighbouring environment constituted 3 x 3 x 3 compartments, resulting in a inspected volume of 180 x 180 x 180 μm surrounding the particular cell.

3 RESULTS

Scaffold Strain Mechanics

The apparent elastic modulus of 392Pa related well to experiments ($\sim 206\text{Pa}$ [Harley *et al.*, 2007] and $\sim 500\text{Pa}$ [Haugh *et al.*, 2009]). As also previously measured ($\sim 80\%$ for dry CG scaffold [Harley *et al.*, 2007]), anisotropy was observed, i.e. E_x and E_y were approximately 60% of E_z . The principal stress distribution within the scaffold was highly heterogeneous (Figure 4a). Distinct stress concentrations (Figure 4a) were also evident, though the mean principal stress of $-27.95 \pm 40.84\text{Pa}$ was relatively close to zero (1% scaffold compression).

Scaffold Fluid Velocities

The mean interstitial fluid velocities within the scaffold relate well to published data. In particular, the mean interstitial velocity for each flow condition was higher than the inlet velocity ($1.11 \pm 0.79 \mu\text{ms}^{-1}$, $11.12 \pm 7.88 \mu\text{ms}^{-1}$ and $111.22 \pm 78.88 \mu\text{ms}^{-1}$ for an inlet of 1, 10 and $100 \mu\text{ms}^{-1}$, respectively); Jungreuthmayer *et al.* (2008) found a mean interstitial velocity of $296 \mu\text{ms}^{-1}$ for an inlet of $235 \mu\text{ms}^{-1}$. Figure 4b illustrates the heterogeneity of the fluid velocities ($1 \mu\text{ms}^{-1}$ inlet velocity).

Cell Growth

A total cell number of $\sim 4.20 \times 10^6$ cells/cm³ proved consistent for many of the scaffold strains and inlet fluid velocities (Figure 5i-vi). However, for an $100 \mu\text{ms}^{-1}$ inlet velocity, cell numbers were greatly reduced to a relatively low density of $\sim 1.30 \times 10^6$ cells/cm³ for scaffold strains of 1 and 2% compression, while at a strain of 5%, the density only reached $\sim 0.69 \times 10^6$ cells/cm³ (Figure 5vii-ix).

The rate of total cell numbers began with a toe-region before quickly increasing linearly and before levelling out just below the respective maximum density (Figure 5i-vi). Interestingly, the proliferation for each phenotype was not consistent. In fact, MSC proliferation displayed a substantial decrease immediately following a peak (Figure 5i-vi), while the SPC phenotypes demonstrated a lesser increase in cell number, yet depending on specific combinations of stimuli, did however tend to plateau at a particular cell density (Figure 5i-vi).

MSC differentiation showed a phenotype preference to specific combinations of scaffold strains and inlet fluid velocities (Figure 6). In particular, for the lowest combination of scaffold strain (1% compression) and scaffold inlet velocity ($1 \mu\text{ms}^{-1}$), OST cells dominated the differentiation lineage (84.9% of total cell number); see Figure 7c. For a 5% scaffold strain and a $10 \mu\text{ms}^{-1}$ inlet velocity, FBT cells were the majority phenotype (73.9% of total cell number); see Figure 7a. However, unlike its counterparts, there was no explicit combination of scaffold strain and inlet velocity that encouraged solely CHD cells. The most suitable combination of stimuli arose from 5% scaffold strain and $1 \mu\text{ms}^{-1}$ inlet velocity (56.7% CHD); see Figure 6iv and 7b.

MSC differentiation also elicited phenotype preferences for specific regions (Figure 8). While the air-side negatively influenced OST cells, the intermediate regions demonstrated positive effects for both FBT and CHD cells, though interestingly, all cells were negatively influenced by the pan-side.

4 DISCUSSION

This investigation predicted cell growth within a CG scaffold for a range of scaffold strains and inlet fluid velocities. As a result, the simulations were able to propose cell-specific preferences to particular combinations of CG scaffold loads.

A rigorous validation procedure that allows multiple phenotypic markers to be identified and quantified within a mixed cell population is not available. Thus, unfortunately, there are no data or experimental methodologies available to directly validate the results of this investigation. Consequently, the findings in this investigation are purely predictive. However, despite this limitation, every cell parameter, albeit a differentiation potential or a biophysical stimulus, was calibrated with published experiments. Thus, as each and every step was calibrated to experimental measurements, the system of methodologies employed herein could be considered reasonably meaningful; in the subsequent sections, published data are employed to assess the physical appropriateness of the results.

The sample size ($n=1$) used in this study was small. However, previous work by one of the authors (O'Brien *et al.*, 2004) found a very small variance (0.5%) in the microstructure across multiple samples of CG scaffolds (achieved by comparing pore size characteristics). In fact, the fabrication technique presented by this 2004 paper (constant cooling rate lyophilisation) was developed in order to provide a method that minimised the microstructural variance between samples. As only a 0.5% variance is apparent between multiple samples, then the use of a single sample can be considered representative of this type of scaffold. Furthermore, other authors have also noted this relative uniformity between CG scaffold samples [Harley *et al.*, 2007, Harley *et al.*, 2008].

By modelling the cells as triangular elements, cell representation was limited to one morphology. However, as 76% of cells within a CG scaffold are attached across multiple struts [Stops *et al.*,

2008], the implementation of a geometry that spread over multiple struts (cells were attached by 3 strut nodes), could be considered reasonably representative. Furthermore, the passive nature of the modelled cells omitted the contractile property that has been shown to impart bending forces on struts [Harley *et al.*, 2008]. Primarily, in justification, the complexity of incorporating an interactive component in the FE and CFD simulations proved too computationally expensive (the simulations contained 6.4 and 40.1 million elements respectively). However, as cells contract in order to distort the scaffold and create a strain-rich environment [Freyman *et al.*, 2002], then as the modelled scaffold was loaded, it is likely that cells seeded in this already highly-strain-rich environment would not seek to actively distort the scaffold.

Analysis of Results

Cell growth was highly influenced by CG scaffold loading. In fact, for scaffold strains of 1-5% compression with inlet fluid velocities of $1\text{-}10\mu\text{ms}^{-1}$, the cells proliferated to such an extent that the maximum cell density was almost achieved (Figure 5-7). However, in stark contrast was the fast inlet velocity of $100\mu\text{ms}^{-1}$, which influenced cell growth so significantly that along with a scaffold strain of 5% compression, almost no proliferation occurred. This finding can be explained by the mean interstitial fluid velocities. For an inlet velocity of $100\mu\text{ms}^{-1}$, the mean interstitial velocity was $111.22\pm 78.88\mu\text{ms}^{-1}$, which is above the $105.471\mu\text{ms}^{-1}$ threshold set by the proliferation algorithm (based on experimental measurements). Interestingly, previous experiments [Cartmell *et al.*, 2003] have shown inlet fluid velocities above $53.90\mu\text{ms}^{-1}$ to decrease cell viability, while very fast inlet velocities above $235\mu\text{ms}^{-1}$ cause cell necrosis [Jaasma and O'Brien, 2008]. It is likely that these fast inlet velocities impart shearing forces that either detach the seeded cells from the scaffold, or disrupt cell integrity. Consequently, to encourage cell proliferation, it appears that inlet velocities should afford mean interstitial velocities below $105.471\mu\text{ms}^{-1}$.

For the biophysical stimuli shown in Figure 6 (1-5% scaffold strain with $1\text{-}10\mu\text{ms}^{-1}$ inlet fluid

velocities), the proliferation of the total cell number followed experiments [Ignatius *et al.*, 2005; Kaspar *et al.*, 2002; Wang *et al.*, 2005]. Interestingly, these six simulations all demonstrated a peak cell density of $\sim 4.20 \times 10^6$ cells/cm³, $\sim 90\%$ of the maximum 4.63×10^6 cells/cm³. These densities concur with CG scaffold experiments ($\sim 1.6 \times 10^6$ cells/cm³ seeding density [Tierney *et al.*, 2009])

For a combination of 1% scaffold strain with $1 \mu\text{ms}^{-1}$ inlet velocity, OST cells dominated the array (84.9% of total cell number). This suggested that bone-like tissue may form under these conditions. Interestingly, at low fluid velocities and low shear strains, Søballe *et al.* (1992a, b) and Søballe (1993) also found significant OST proliferation within a fracture callus. Under a combination of 5% scaffold strain with $10 \mu\text{ms}^{-1}$ inlet velocity, FBT cells dominated the cell assortment (73.9%). Again Søballe *et al.* (1992a, b) and Søballe (1993) observed a similar pattern: fast fluid velocities and high shear strains encouraged FBT. Interestingly, a predominance of CHD was not found. Yet for 5% scaffold strain with $1 \mu\text{ms}^{-1}$ inlet velocity, CHD comprised the majority of the total cell number (56.7%), but had significant contributions from OST and FBT (26.2% and 13.3% respectively). This suggested high octahedral shear strain with low fluid velocity encourages cartilage-type growth. However, CHD cells also contributed largely at 1% scaffold strain with $10 \mu\text{ms}^{-1}$ inlet velocity: FBT cells had 55.6% and CHD 41.3%. As a result, this suggested low octahedral shear strain with relatively high fluid velocity encourages cartilage-type growth. Though not the majority phenotype in this latter case, CHD cells do appear to favour a range of biophysical stimuli. Simply, these two assortments might be classified as osteo-cartilaginous and fibro-cartilaginous tissues.

Interestingly, regional phenotype preference was observed within the CG scaffold. In particular, trends indicated that the pan-side had a negative effect on all phenotypes (Figure 8). However, the lowermost and uppermost intermediate regions showed positive effects on FBT and CHD differentiation (Figure 8*i* and *ii*). Conversely, the uppermost portion suggested negative effects on FBT (Figure 8*iii*), though the air-side demonstrated positive effects on OST growth (Figure 8*iv*).

This regional preference can be explained by the fluid profiles. As there were only a few holes that permitted fluid to pass in the thin film-like structure on the air-side (Figure 4b), the air-side had hotspots of high pressure causing a high variance of fluid velocities (peaks and troughs). Conversely, the remainder of the scaffold experienced a relatively low velocity variance due to the more equiaxed pore structure. Consequently, even though the mean velocities are similar, these two portions had very different fluid profiles: the air-side endured high variance in velocity, while the remainder had a low velocity variance. Thus, it is likely that CHD and FBT cells, which are suited to medium velocities, are conducive to the low variance portion, while OST cells, which are suited to very low velocities, may suit the ‘troughs’, or the low hotspots, in the air-side portion. What effect this mechanism would have if the scaffold is orientated differently in the bioreactor is unknown, but it does suggest that architecture plays an important role in MSC differentiation.

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Conflict of Interest Statement

No conflicts of interest.

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FIGURES

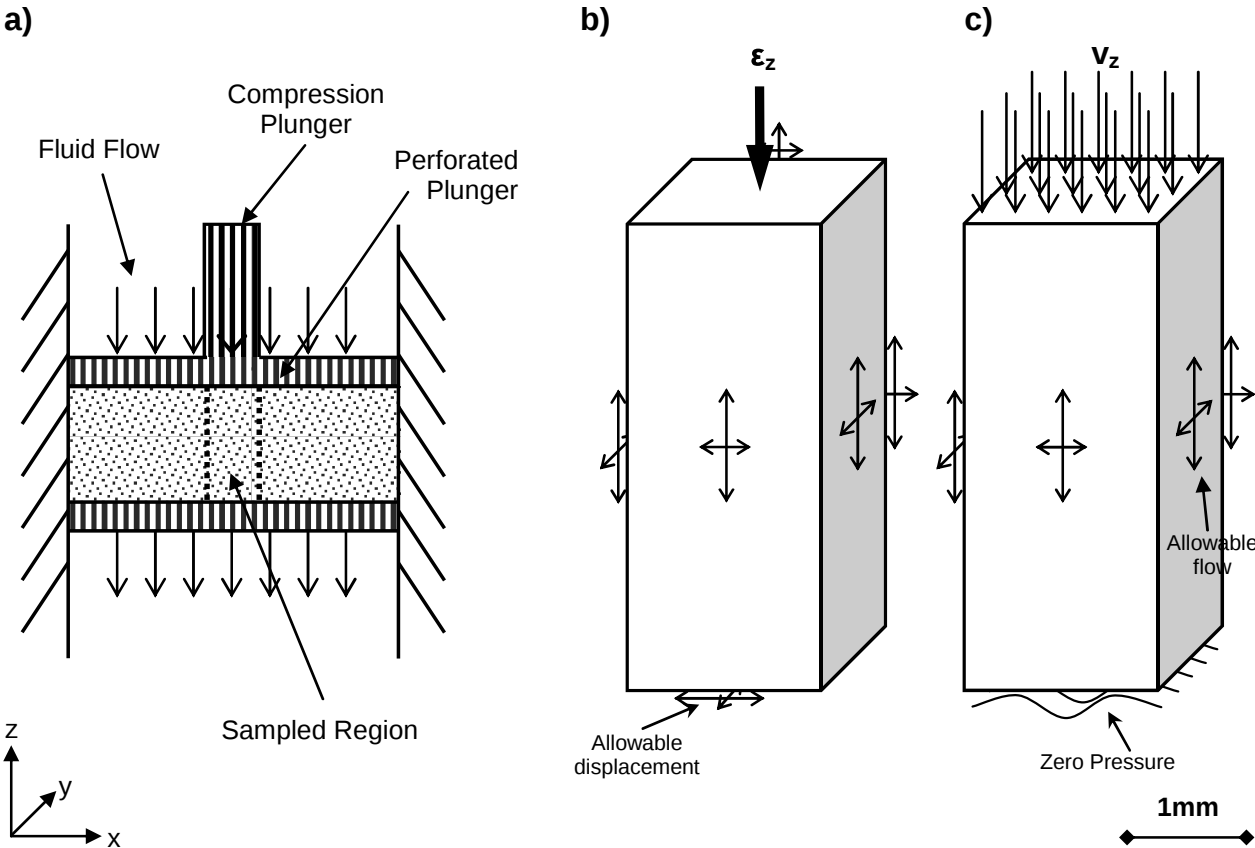


Figure 1. The FE and CFD models were developed in order to simulate a typical perfusion bioreactor: a) is a schematic illustration of the Shulz *et al.* (2008) bioreactor, while b) depicts the corresponding boundary conditions applied to the FE model, and c) shows the appropriate boundary parameters implemented in the CFD model.

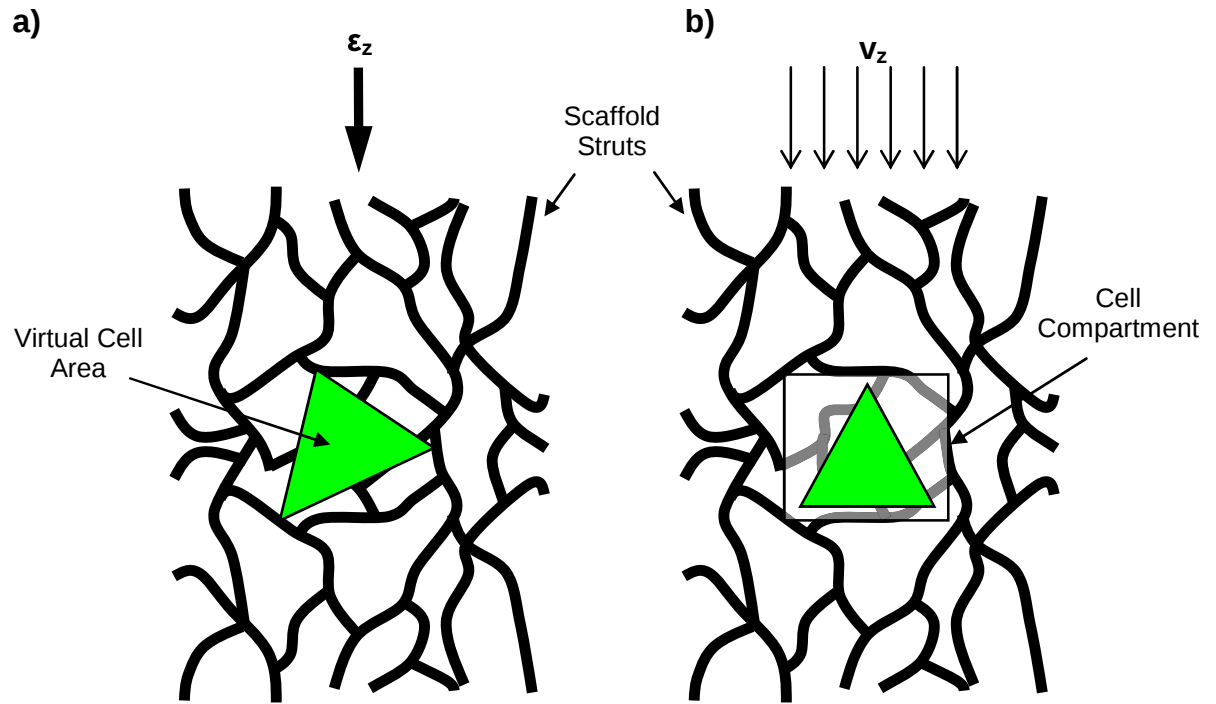
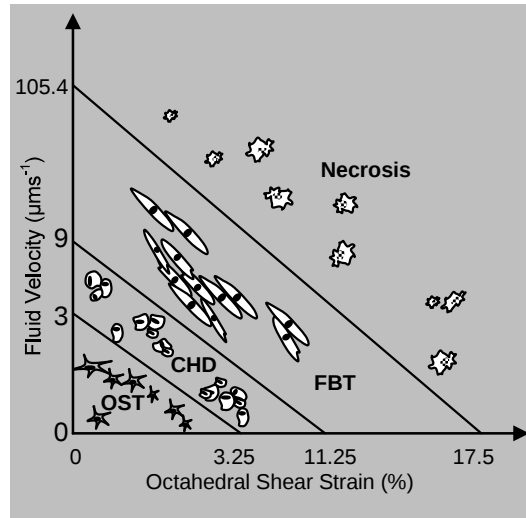


Figure 2. The biophysical stimuli experienced by the cells were calculated in two forms: a) by the deformation of a virtual area which was defined from three nodes on the scaffold struts, and b) by the mean fluid velocity within a 60 x 60 x 60 μ m compartment. Note the above is a schematic representation of the modelled system.



Modified Prendergast *et al.* (1997) Mechano-Regulatory Model (cell necrosis added)

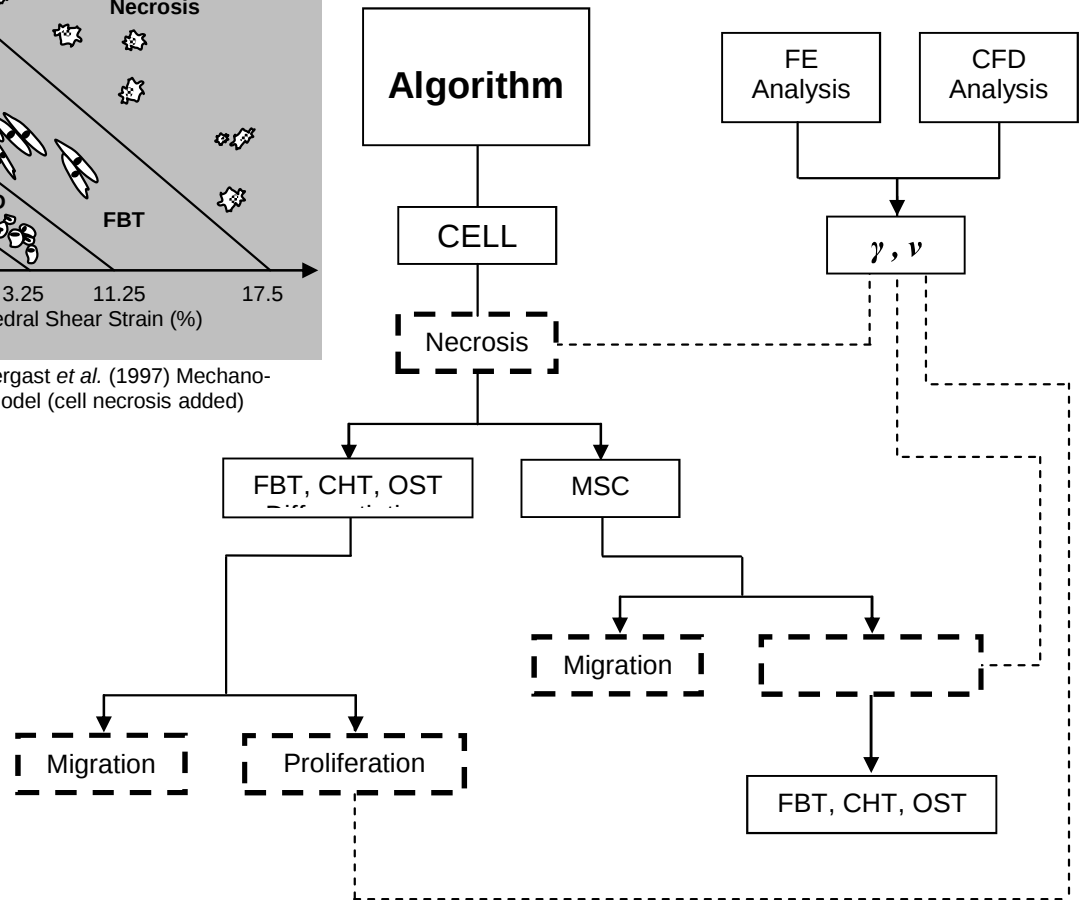


Figure 3. The cell algorithm simulated proliferation, differentiation, migration and necrosis. Biophysical stimuli, as determined from the FE and CFD analyses, determined cell phenotype following MSC differentiation, proliferation potentials for specialised cells, and necrosis for all cells. The text framed by thick dashed lines (— —) indicate decision processes within the algorithm, while the thin dashed lines (----) signify the input of data. Insert depicts mechanoregulatory algorithm used for MSC differentiation.

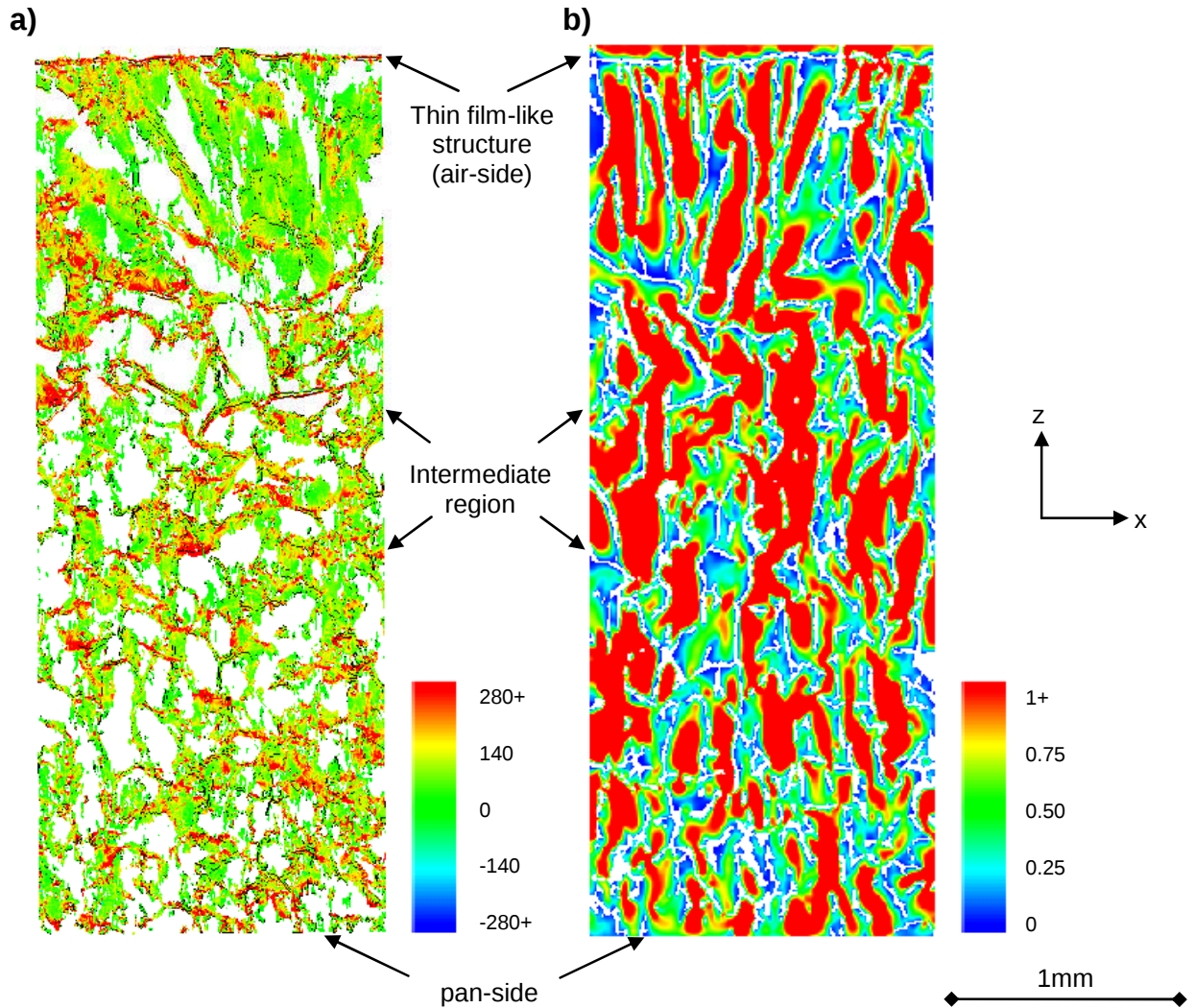
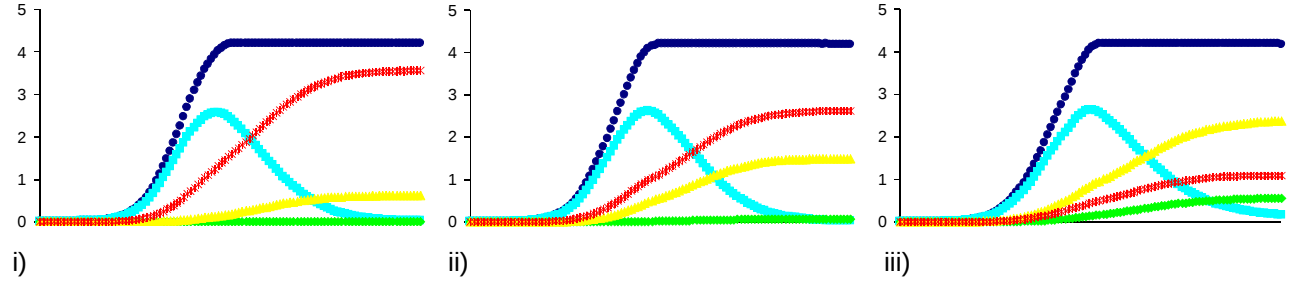
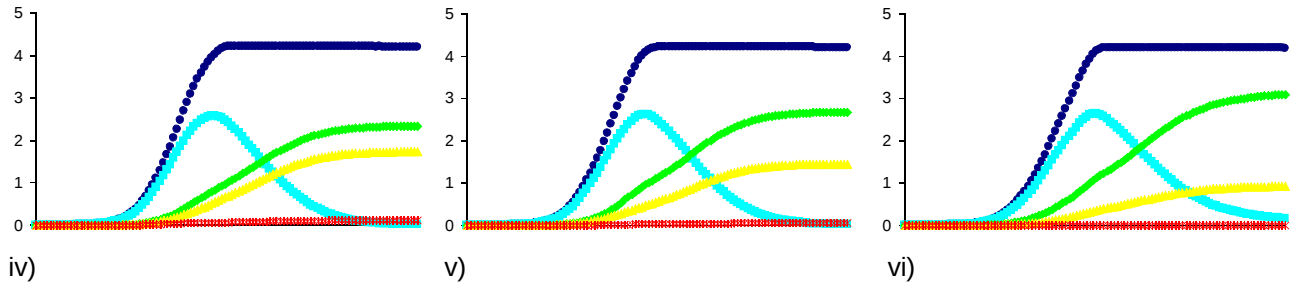


Figure 4. The modelled CG scaffold demonstrated heterogeneous characteristics in terms of mechanical deformation (FE model) and fluid dynamics (CFD model); following a uniaxial compressive strain of 1% in the z-axis, (a) depicts the von Mises stress (Pa) distributions from a 2D slice of the FE mesh, while subsequent to a constant inlet velocity of $1\mu\text{m}\cdot\text{s}^{-1}$, (b) details a 2D slice of the fluid velocity ($\mu\text{m}\cdot\text{s}^{-1}$). Note 2D slices are shown for illustrative purposes only; the models were of a 3D configuration ($1.5 \times 1.5 \times 3.9\text{mm}$).

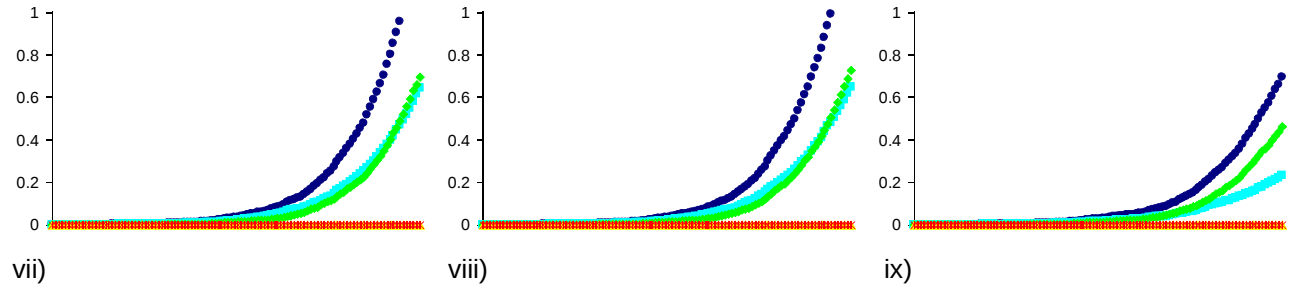
Inlet Fluid Velocity = $1\mu\text{ms}^{-1}$



Inlet Fluid Velocity = $10\mu\text{ms}^{-1}$



Inlet Fluid Velocity = $100\mu\text{ms}^{-1}$



Scaffold Strain = 1%

Scaffold Strain = 2%

Scaffold Strain = 5%

TOTAL MSC FBT CHD OST

● —■— ◆ —▲— *

Figure 5. The proliferation of the cells are shown for the nine simulated regimes; i-iii) show cell responses to $1\mu\text{ms}^{-1}$ fluid inlet velocity with 1, 2 and 5% scaffold strain, iv-vi) $10\mu\text{ms}^{-1}$ fluid inlet velocity with 1, 2 and 5% scaffold strain and vii-ix) depicts the proliferation for $100\mu\text{ms}^{-1}$ fluid inlet velocity with 1, 2 and 5% scaffold strain respectively. Note the horizontal axes correspond to time, while the vertical axes refer to cell density ($\times 10^6$ cells/ cm^3).

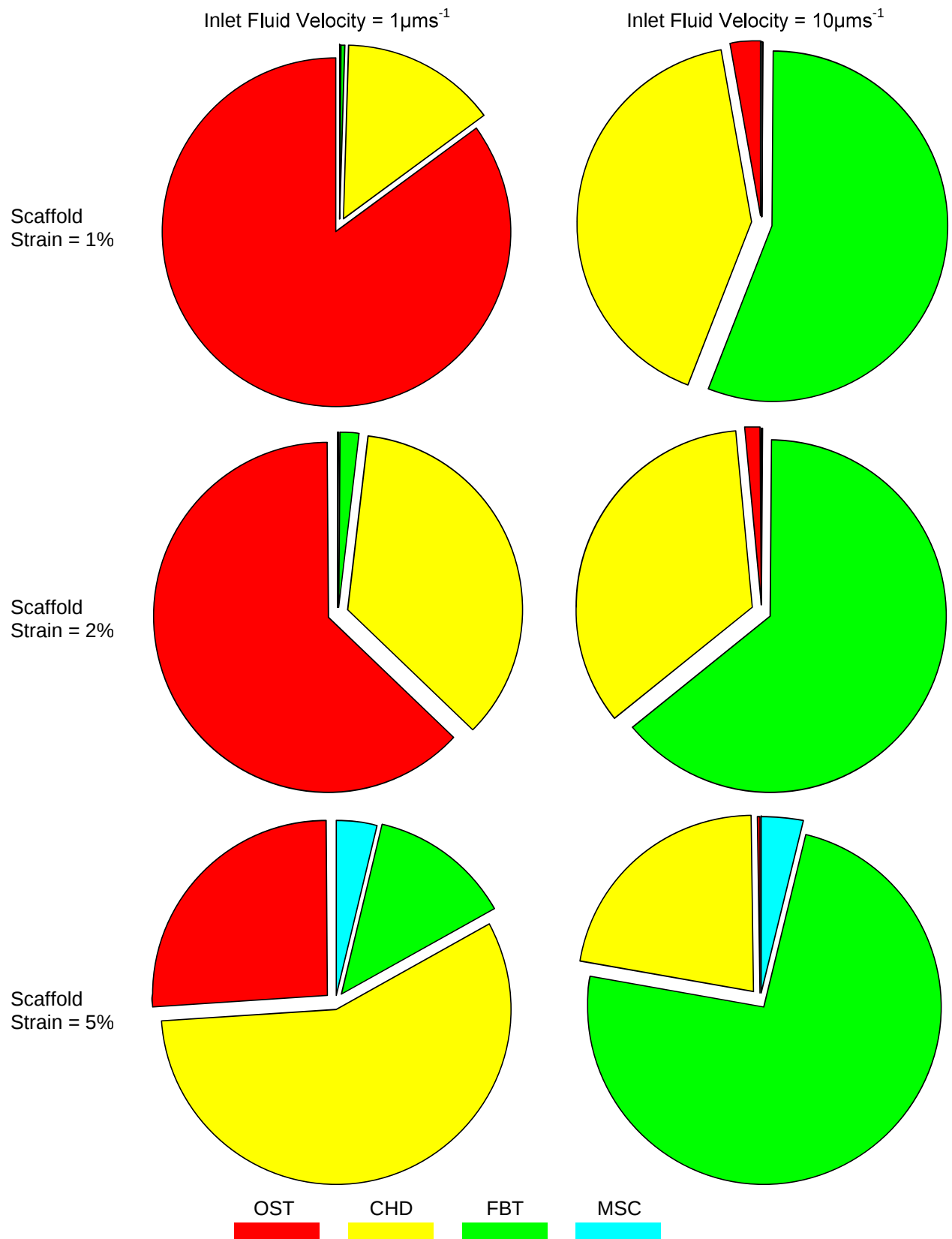


Figure 6. The predicted cell phenotypes within the modelled volume are shown in terms relative to total cell number. Note the annotations referring to strain and fluid velocity denote the respective scaffold model inputs for the corresponding pie chart. Note only 6 models are presented here as the remaining models did not provide sufficient cell densities to allow a statistical comparison.

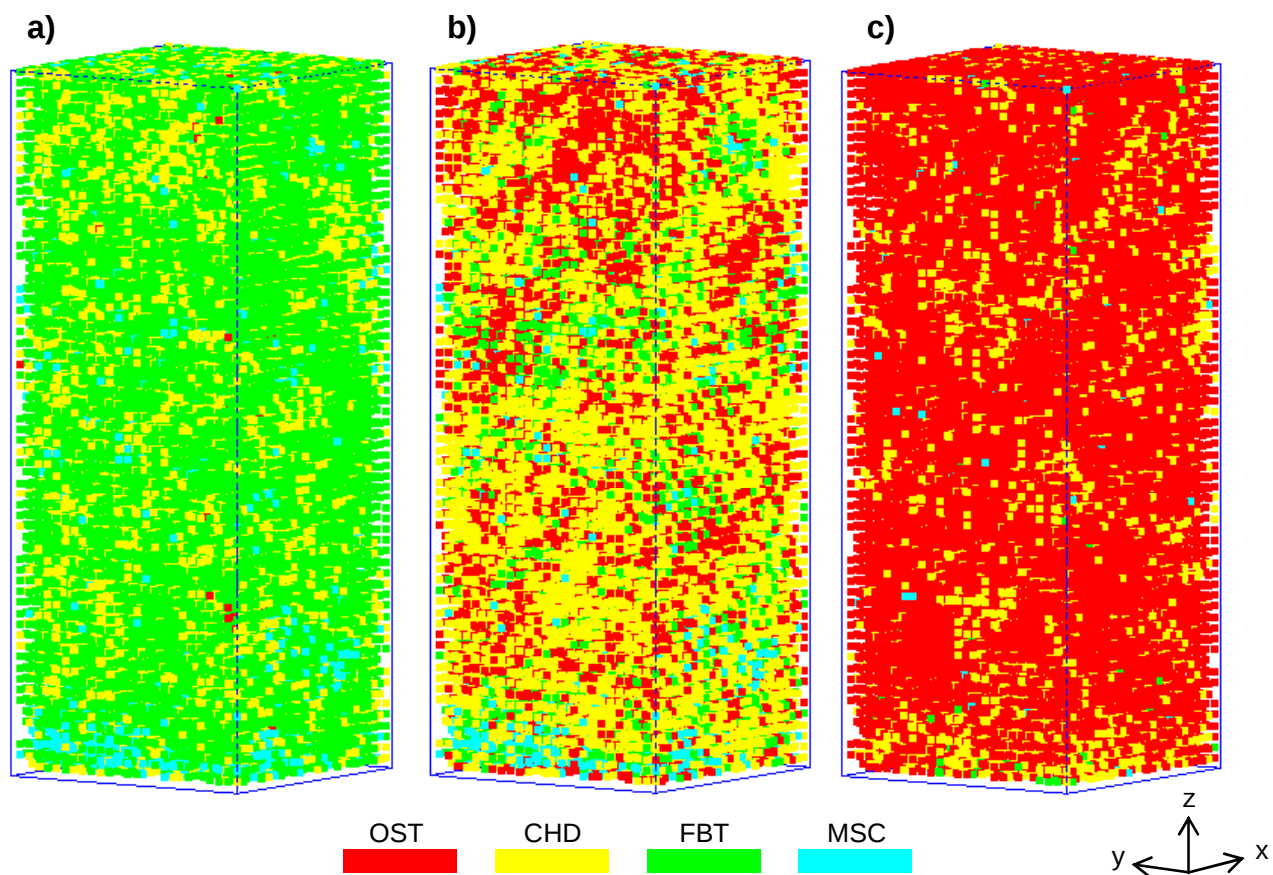


Figure 7. The predicted differentiation patterns within the modelled volume are shown here for a) a FBT dominated volume (where 73.9% of the cells were FBT) which resulted from a 5% scaffold strain and a $10\mu\text{ms}^{-1}$ inlet velocity, b) a volume with mostly CHD cells (56.7% CHD) which arose from a 5% scaffold strain and a $1\mu\text{ms}^{-1}$ inlet velocity and, c) an OST cell majority (84.9% OST) which was a product of a 1% scaffold strain and a $1\mu\text{ms}^{-1}$ inlet velocity.

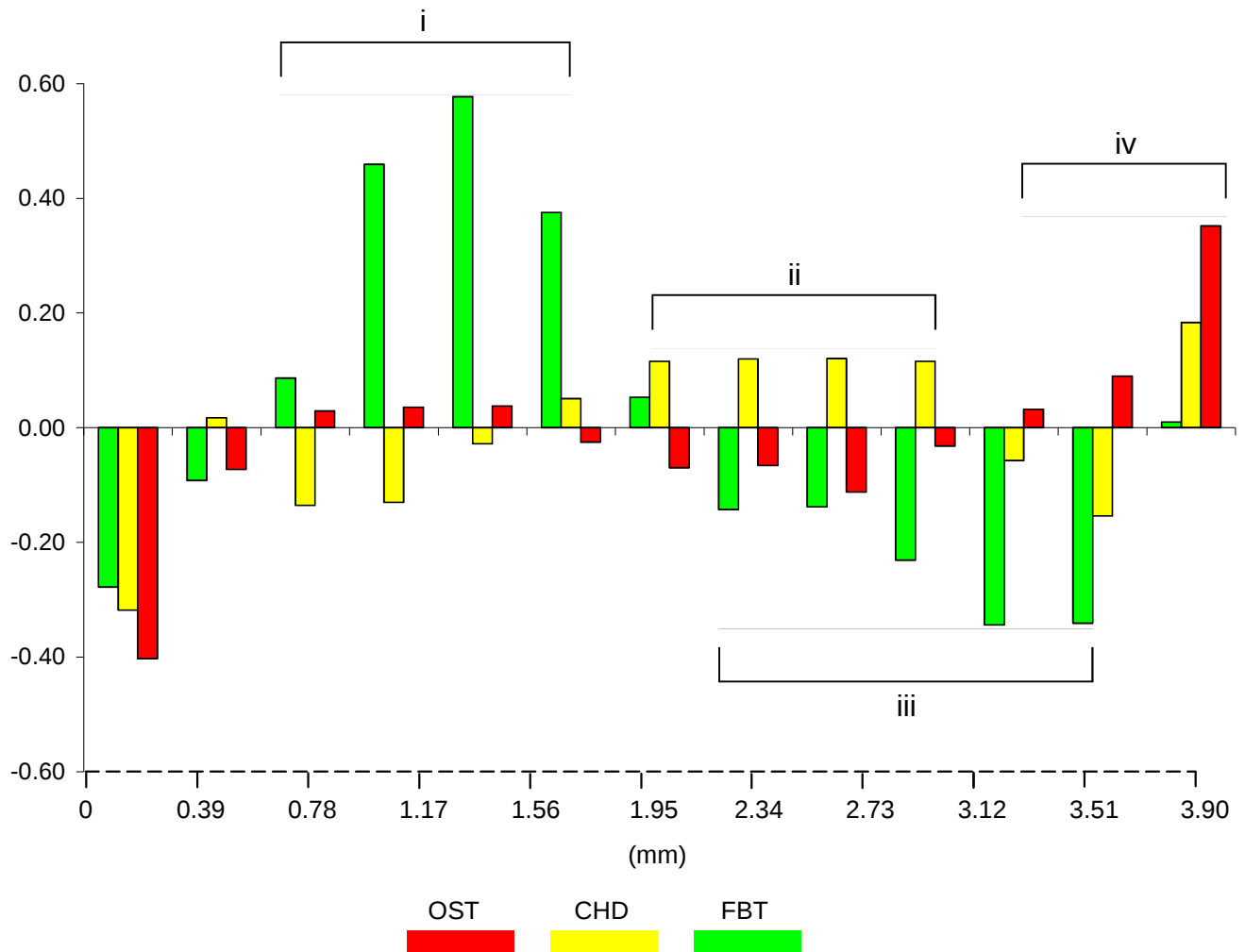


Figure 8. The regional effect on MSC differentiation into specific phenotypes was evaluated using Cohen's effect size methodology [Cohen, 1977]. Indications are made for occurrences of 3 or more contiguous regions with likewise effects above ~ 0.1 : i) shows the lowermost intermediate regions to have a positive effect on FBT growth, ii) the uppermost intermediate regions a positive influence on CHD, iii) the uppermost regions to have a negative effect on FBT, and iv) suggests the air-side regions to have a positive effect on OST differentiation. Note, the horizontal axis refers to the position along the z-axis of the CG scaffold (axis convention in Figure 1), where a position of zero relates to the 'pan-side' while a position of 3.90mm is equivalent to the 'air-side'.

Conflicts of Interest Statement

They are no conflicts of interest to declare by the authors.