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Word Count:

Nomenclature

δ	Engineering strain
θ	Angle of cells orientation to applied stretch
ν	Poisson's ratio
ε	Green's strain
γ	Shear strain
N	Number of data point (50)
F	Cumulative distribution (CDF) for Normal distribution
S	CDF for experimental distribution
D_N	Difference in CDF's
T_{KS}	Kolmogorov –Smirnov test statistic

Subscripts

x,y	Cartesian co-ordinates
aa	Axial direction along cell

Abstract

In vivo, endothelial cells of the coronary artery are constantly exposed to pulsatile shear and tensile stresses. The main aim of this study was to design and build a mechano-transduction rig, which reproduced homogenous strain profiles of the tensile strain experienced *in vivo*, and to investigate the effect of this cyclic tensile strain on the cell morphology, cell orientation and protein expression of endothelial cells. The biological response of human umbilical vein endothelial cells to a uni-axial cyclic stretch, in this newly developed bioreactor, was examined experimentally and compared to a mathematical model. In the model, it was revealed that endothelial cells had a strain threshold value of 2.5% when exposed to a 10% cyclic strain at 1Hz for 3 hours. Following phalloidin staining and confocal imaging to detect actin distribution, it was found that the cells elongated and oriented at 65° to the direction of applied stress. A decrease in VCAM-1 and an increase in ICAM-1 protein levels were observed in response to the cyclic strain compared to those cells cultured under static conditions. These levels of protein are comparable with in vivo levels and thus validate the bioreactor as replicating the physiological environment.

1. Introduction

Atherosclerotic lesion development, a hallmark of atherosclerosis, does not occur randomly within the vasculature but rather prevails at certain sites, associated with high stress and high stretch (Sumpio, 1991), such as discontinuities in the arterial trees, the branches of the coronary arteries and the carotid bifurcation. It has been hypothesized that certain mechanical factors such as pressure induced wall stress and blood flow disturbances are contributing factors to the localization of atherosclerotic lesions (Nerem, 1992, Thubrikar and Robicsek, 1995, Zhao, et al., 2002).

The endothelial cell (EC) layer that lines the entire vascular system is a metabolically active monolayer that regulates a variety of biological responses and physiological functions (Topper and Gimbrone, 1999). Alterations in EC gene and protein expression underpin a number of features characteristic of cardiovascular disease including monocyte-endothelial interactions. Due to the constant pulsatile blood flow through the circulatory system, EC are constantly exposed to a shear and tensile stress (Stenson-Cox, et al., 2004). Various *in vitro* investigations have examined these mechanical stresses both independently and in combination. (Chen, et al., 2001, Davies, 1995, Davies, 1997, Lehoux and Tedgui, 2003, Resnick, et al., 2003, Wang, et al., 1995 Ando and Kamiya, 1996, Ando, et al., 1994, Ando, et al., 1995, Chen, et al., 2001, Davies, 1995, Davies, 1997, Davies and Tripathi, 1993, Flaherty, et al., 1972, Florian, et al 2003, Gimbrone, et al., 1997, Gimbrone, et al., 2000, Kano, et al., 2000, Korenaga, et al., 1997, Mohan, et al., 2003, Nerem, 1992, Nerem and Girard, 1990, Qui, et al 2000, Sumpio, 1991, Topper, et al., 1996, Topper and Gimbrone, 1999, Tsao, et al., 1996, Tsuboi, et al., 1995, Wang, et al., 2000, Wasserman, et al., 2002). However, these studies have been carried out on

endothelial cells from a variety of sources using a wide range of physiological parameters, including various physiological testers, frequencies and strain rates. Due to inconsistent testing materials and methods, it is difficult to compare and extrapolate the results obtained from one experimental study to another.

Although the biological response of endothelial cells subjected to fluid flow has been extensively studied, the biological response of endothelial cells on deformable substrates subjected to cyclic strain still requires further characterisation. Many of the studies seed cells on circular substrates to which a vacuum is applied (Cheng, et al., 1996, Riser, et al., 1999, Yun, et al., 1999). A shortfall of such studies means that the cells are exposed to a heterogeneous strain field with minimum strain at the centre of the membrane and a maximum strain at the periphery. Cell morphology and cell orientation observed on these circular substrates tend to be more heterogeneous due to the nature of the strain applied (Chen, et al., 2001, Riser, et al., 1999, Yun, et al., 1999).

This study aims to address some of these issues by employing finite elelment analysis (FEA), biomechanical engineering principles and biological techniques to design, build and validate a physiological simulator for pulsatile stretch investigations. Using FEA, the strain profile of the circular substrates outlined above was evaluated and compared to rectangular strips. Using these analyses, a physiological simulator capable of exposing rectangular substrates to *in vivo* levels of pulsatile strain was developed. Mathematical modelling was employed to predict the effect of the cyclic stretch on the cellular response. To validate the system, cell morphology and cell orientation was examined and compared to the mathematical

model. An added dimension to this research was the use of molecular biology techniques to validate the system by comparing the cell response on the simulator to the cellular response experienced *in vivo*.

2. Materials & Methods

2.1 Finite element analysis

Finite element analysis was used to investigate the actual strain profiles on circular deformable substrates used in previous studies. FEA was then used to design a substrate which would give a uniform strain profile when uni-axial strain was applied to it. This way it can be ensured that the substrate shape chosen has a homogeneous strain field.

2.2 Design and development of mechano-transduction rig

The basic working principle of the mechano-transduction rig developed to evaluate endothelial cell response to uni-axial cyclic stretching is shown in Figure 2 and consists of the cell-seeded silicone substrate, clamped at both ends, is stretched by moving one of the clamps, relative to the other fixed clamp, along the long axis of the silicone membrane. The rig itself was made from PMMA, a biocompatible transparent material that allowed visualization of the test. The fixed grip of the rig was designed so that it was self-tightening upon loading and would not loosen during testing. The rig was easily mounted into the environmental chamber of an Instron 8874 tensile testing machine, where cyclic conditions were applied to the cell seeded silicone system in a precisely controlled, recordable and repeatable manner. The environmental chamber provided the required physiological conditions during the test.

During the test the cell-seeded silicone substrates were subjected to a 10% cyclic strain at a frequency of 1 Hz for 3 hours in phosphate buffered solution at 37°C in the environmental chamber.

2.3 Cell culture and treatment

Studies were performed using human umbilical vein endothelial cell (HUVEC) obtained from Cambrex, UK. Cells were maintained in HUVEC EBM-2 Bullet kit media (Cambrex, UK). Cells were propagated at 37°C, in a humidified incubator with a 5% CO_2 air atmosphere and were used at passages 2-5. The silicone strips 12.5mm X 50 mm X 1.0 mm were sterilized using an ultra violet source for 1 hour. The cells were seeded onto the strips at a density of 40,000/cm^2 under sterile conditions and were subjected to either a 10% cyclic strain at a frequency of 1 Hz for 3 hours or to a 0% strain in the mechano-transduction rig (37°C, humidified incubator, 5% CO_2). To examine the effect of physiological stretch alone on cell behaviour, cells were grown to subconfluency to avoid cell - cell interactions. The sampling process for the statistical analysis involved selecting regions containing 50 cells with few cell-cell interactions (Neidlinger-Wilke, et al., 2001).

The substrate upon which the cells are seeded plays a very important role when applying the cyclic stretch. The chosen substrate has to be elastic when subjected to cyclic stretch, be biocompatible, non-toxic to cells and have suitable surface properties for efficient cell attachment. The material used in this study was a commercially available silicone elastomer (Good Fellow, U.K.) with a Youngs modulus of 1.8 MPa, a tensile strength of 6.5 MPa and a percentage elongation at

break of 300%. Prior to cell seeding, silicone substrates were plasma treated in air for 10 minutes using 150 Watts in a Europlasma Junior Plasma Treatment chamber.

2.4 Statistical Methods

In a previous study, (Wang, et al., 1995) states that the cell axial strain (ε_{aa}), varies with the angle (θ) to the applied stretch (Figure 3a) and Poisson's ratio (ν) as:

$$\varepsilon_{aa} = \varepsilon_{xx}\cos^2(\theta) + \varepsilon_{yy}\sin^2(\theta) + \frac{\gamma_{xy}}{2}\sin(2\theta) \tag{1}$$

For the experiments of (Wang, et al., 1995) and for the experiments reported here, x is the substrate stretch direction and y is the transverse substrate contraction direction. For this choice of axes and loading, the shear strain γ_{xy} is zero. Also ε_{xx} and ε_{yy} are the Green's (large) strain components given by:

$$\begin{aligned}\varepsilon_{xx} &= \delta_x + \delta_x^2/2 \\ \varepsilon_{yy} &= \delta_y + \delta_y^2/2\end{aligned} \tag{2}$$

where δ is the engineering strain. Also, from the definition of Poisson's ratio:

$$\delta_y = -\nu\delta_x \tag{3}$$

Using the experimental values of δ_x equal to 0.1, a Poisson's ratio of 0.35 and a zero shear strain, the distribution of axial strain is determined from equation (1) for a range of cell orientations and shown in Figure 3b. From this plot, it can be seen that a minimum value of axial strain occurs at 63°, where the cells experience a net strain of zero along their axial direction.

2.5 SEM and Immunocytochemistry studies

Cell morphology and orientation was examined using scanning electron microscopy (SEM) and confocal laser scanning microscopy (Zeiss LSM 510)

(CSLM). Immediately after mechanical stimulation or static conditions endothelial cells were fixed and stained for analysis described below. For SEM examination the endothelial cells were fixed with gluteraldehyde and dehydrated with increasing concentrations of ethanol up to 100% and dried using critical point drying. The cells were then coated with a thin layer of gold to permit conductivity of the sample for SEM examination. Fluorescent conjugates of phalloidin (Molecular Probes, UK) were used to label the actin filaments for examination of cell morphology and cell orientation. Propidium iodide (Molecular Probes, UK), which binds to cellular DNA and RNA was used to visualise the nucleus, while FITC conjugated mouse anti-human ICAM-1 and VCAM-1 monoclonal antibodies (TCS Cellworks, UK) were used to stain for protein expression of the aforementioned adhesion molecules. Additionally, analysis of the expression of ICAM-1 and VCAM-1 was conducted using Image Pro® Plus analysis software (Media Cybernetics, UK) where the protein expression was visually quantified by measuring the area of green fluorescence present, representing each adhesion molecule, compared to the area of the nucleus of the cell.

3. Results

3.1 Finite Element Analysis

In this study it has been shown through finite element analysis that the actual strain profile on circular deformable substrates used in previous studies (Cheng, et al., 1996, Riser, et al., 1999, Yun, et al., 1999) varies significantly from the average profile. Radially the percentage difference ranges from –28% to 31% and circumferentially from – 42% to + 62% shown in Figure 1.

The substrate shape, which would ensure a homogenous strain profile, is a rectangular shaped substrate, which has been implemented into this rig design. *<<<Refer to Karen's rectangular substrate strain profile results>>>*

3.2 Cell Morphology

As mentioned previously the cell orientation and cell morphology was examined using SEM and CLSM. The direction of stretch is indicated by the arrow to the right of the images (Figure 4-6). The images obtained using SEM reveal the general outline of cell orientation and cell morphology (Figure 4), while the images obtained from the CLSM revealed more details in terms of the orientation and morphology of the nucleus (Figure 5) and the actin filaments of the cell (Figure 6).

From the SEM images in Figure 4a it can be seen that the cells grown in static culture were randomly oriented and had cobblestone morphology. However, the cells subjected to cyclic stretch (Figure 4b) were seen to elongate and realign obliquely to the direction of stretch. This is further evidenced in Figure 5 in propidium iodide stained CLSM images, where it can be seen that the nuclei of the cells grown in static culture were randomly oriented (Figure 5a), while Figure 5b reveals that the nuclei of the stretched endothelial cells begin to elongate and realign at an angle to the direction of stretch.

This is also highlighted in the phalloidin stain for the actin filaments of the cytoskeleton. The actin filaments of the endothelial cells were also seen to respond to the cyclic stretch. Compared to the randomly oriented actin filaments in the cells grown in static culture (Figure 6a), the actin filaments of the stretched cells were

remodelled into stress fibres which oriented obliquely to the direction of stretch, as shown in Figure 6b.

3.3 Cell Orientation

To study the effect of cyclic stretch on cell reorientation specifically, the endothelial cell orientation distributions were obtained using statistical analysis, and are shown in Figure 7a and 7b. This was conducted by manually tracing the perimeter of each cell and drawing the major axis of the acquired shape. The orientation angle was found by measuring the angle between the major axis and the baseline, represented by the stretching direction. The acute angle between the perpendicular baseline and the major axis of the cell was recorded and all results were between 0° and 90°. An angle of 0° was indicative of cell aligned perfectly parallel to the stretching direction, while an angle of 90° was indicative of the cell aligned perfectly perpendicular to the stretching direction. From these cell orientation measurements, cell frequency distributions were calculated and are shown in Figure 7. As shown in Figure 7a, the orientation of the mechanically conditioned endothelial cells was in the range 62.5° to 67.5° with a peak at 65°, while a random orientation was observed for cells grown in static culture (Figure 7b).

It should be noted also that 80% of the cells measured fall between an orientation of 50° and 80° (Figure 7a), this gives a threshold strain of 0.025 as can be seen from the solid line in figure 3b for the axial strain analysis. Further, 96% of the cells measured fall within 50° to 90°, this corresponds to a standard deviation for the axial strain of 0.01 as can be seen from the broken line in figure 3b.

3.4 Statistical Analysis

The cell orientation can also be expressed by equation (4) which is derived by setting the shear strain term in equation (1) to zero and re-arranging to give:

$$\theta = \cos^{-1}\left(\sqrt{\frac{2(\varepsilon_{aa}/\delta_x) + (2v - v^2)\delta_x}{2(1+v) + (1-v^2)\delta_x}} \right) \tag{4}$$

By assuming that θ follows a normal distribution, with a mean axial strain of 2.5% and a standard deviation of 1% a sample of ε_{aa} was generated. This was achieved by using fifty random numbers on [0,1] and inverting cumulative distribution function. These values were then replaced in equation (4) and a distribution of cell orientation obtained as seen in figure 7c.

To determine if the measured distribution of the cells follows a normal distribution, a Kolmogorov - Smirnov test is performed. In this case D_N is defined as:

$$D_N = max|S_N(\theta) - F(\theta)| \tag{5}$$

Here, S_N is the cumulative distribution function of the measured N data points (N=50) and F is the cumulative distribution function for the normal distribution. In this study, the maximum difference in the cumulative distribution function (D_N) for the measured angular position and the theoretical one is 0.27 (Figure 7d). As both distributions have 50 samples a test statistic D* was obtained for the Kolmogorov – Smirnov two sample test of:

$$D^* = N_1 N_2 D_N = 50.50(0.27) = 675 \tag{6}$$

Using Neave, 1979, this is not significant at the $\alpha 2 = 5\%$ level, as the critical region is D* > 700. The D* obtained corresponds to an $\alpha 2$ value of approximately 7.5%. The difference between the values predicted and the values measured may be due to operator dependence and instrument control used to measure cell orientation.

3.5 Protein Expression

ICAM-1 protein levels are increased in endothelial cells that were cyclically stretched compared to the control samples grown in static culture (Figure 8). In contrast to the CLSM image representing ICAM-1 expression a decrease in VCAM-1 protein expression was observed in cyclically stretched cells as compared to those cultured under static conditions (Figure 9).

Using Image Pro analysis software to examine the amount of green fluorescence, an increase of 11.5 % in ICAM-1 was determined for the cyclically stretched cells, while a 13 % decrease in the expression of VCAM-1 was observed for the mechanically conditioned cells compared to control cells in static culture. Using this method it is not possible to conduct a statistical analysis, but the resolution of the camera for image analysis and the sensitivity of the image analysis software is accurate to within 0.5% error, which confirms the trend observed.

4. Discussion

In this study, finite element analysis was used to validate a homogenous strain profile for a mechano-transduction rig, which was designed and built, capable of mimicking the physiological strain profiles *in vivo*. This rig was used to expose endothelial cells, which were successfully cultured on modified silicone substrates, to cyclic stretching similar to that found in the native artery. Herein, cell orientation was predicted to be in the region of 63° using axial strain analysis, in the range 60.5° to 64.5° using the computer simulation and experimentally was found to be 65° from the vertical, thus echoing the predictions of McGarry, et al., 2004 for endothelial cells.

To develop a deeper understanding of the role of mechanical stimulation on cellular response, various computational models and experimental studies have been conducted to explain the role of mechanical stimulation on cellular response (Moretti, et al., 2004, McGarry, et al., 2004, Neidlinger-Wilke, et al., 2001, Wang, et al., 1995). Wang, et al. 1995 developed a model which revealed that at 12% strain, melanocyte cell orientations tended toward a normal distribution with the greatest number of cells orienting at 60° to the direction of stretch, while at lower strain levels of 4% and 8% the cells were found to exist in a more random distribution.

Wang, et al., 1995 suggested that this cell orientation response could be explained by the cells attempting to reduce their axial deformation to a level below the axial substrate strain threshold. For melanocytes a mean strain threshold was determined to be 3.5% (Wang, et al., 1995). In a separate study, Neidlinger-Wilke, et al., 2001 used a similar model and found a strain threshold for cell orientation of 6.4% for osteoblasts and 4.2% for fibroblasts. In this study it was revealed that endothelial cells had a strain threshold value of 2.5% when exposed to a 10% cyclic strain at 1Hz for 3 hours, thereby providing a further insight into the cellular response of endothelial cells to physiological levels of cyclic strain.

Previous studies suggested that the reorientation response of the cell may have an effect in inducing gene expression (Dancu, et al., 2004) and in directing cellular mRNA levels and protein synthesis (Carver, et al., 1991, Leung, et al., 1976, Wang, et al., 1995). Application of shear stress to cells *in vitro* has been shown to regulate the expression of adhesion molecules including vascular adhesion molecule-1 (VCAM-1)

and intracellular adhesion molecule-1 (ICAM-1) (Chen, et al., 2001, Lelkes, et al., 1998, Sprague, 1999, Topper and Gimbrone, 1999, Yoshisue, et al., 2002). In contrast to shear stress, the effect of cyclic tensile stretch on the expression of adhesion molecules in EC is less defined. Evidence from other studies suggests a selective sensitivity of EC adhesion molecules to cyclic radial strain applied *in vitro* (Cheng, et al., 1996, Yun, et al., 1999). While there was no change in levels of VCAM-1 expression in EC subjected to the heterogenous radial strain profiles discussed earlier, there was an induction in ICAM-1 expression for these cells compared to cells under static conditions (Yun, et al., 1999). The application of a homogeneous strain profile to the cells, the use of scanning electron microscopy, molecular biology staining techniques and confocal laser microscopy to examine the cell morphology and cell orientation analysis is an exciting dimension to this study. Qualitatively, it can be observed that there is an increase in ICAM-1 and a decrease in VCAM-1 observed for the cells exposed to 10% strain at 1Hz for 3 hours compared to controls in static culture.

Using the engineering approach of combining finite element analysis, mechanical design and biological evaluation, this data gives a more representative view of the effect of *in vivo* mechanical strain on the biological response of cells compared to those observed in radial strain test systems (Cheng, et al., 1996, Riser, et al., 1999, Stenson-Cox, 2004, Yun, et al., 1999). In summary, these results provide a fundamental insight to the effect of physiological levels of strain on the biological response of endothelial cells and have important implications for hypertension and atherosclerosis studies.

Acknowledgements

List of Figures

Figure 1

Percentage Difference of Actual Strains to Average Strain

Figure 2

Dynamic Load

Grip

Fixed

Medium

Chamber at
37°C

EC seeded on Silicone
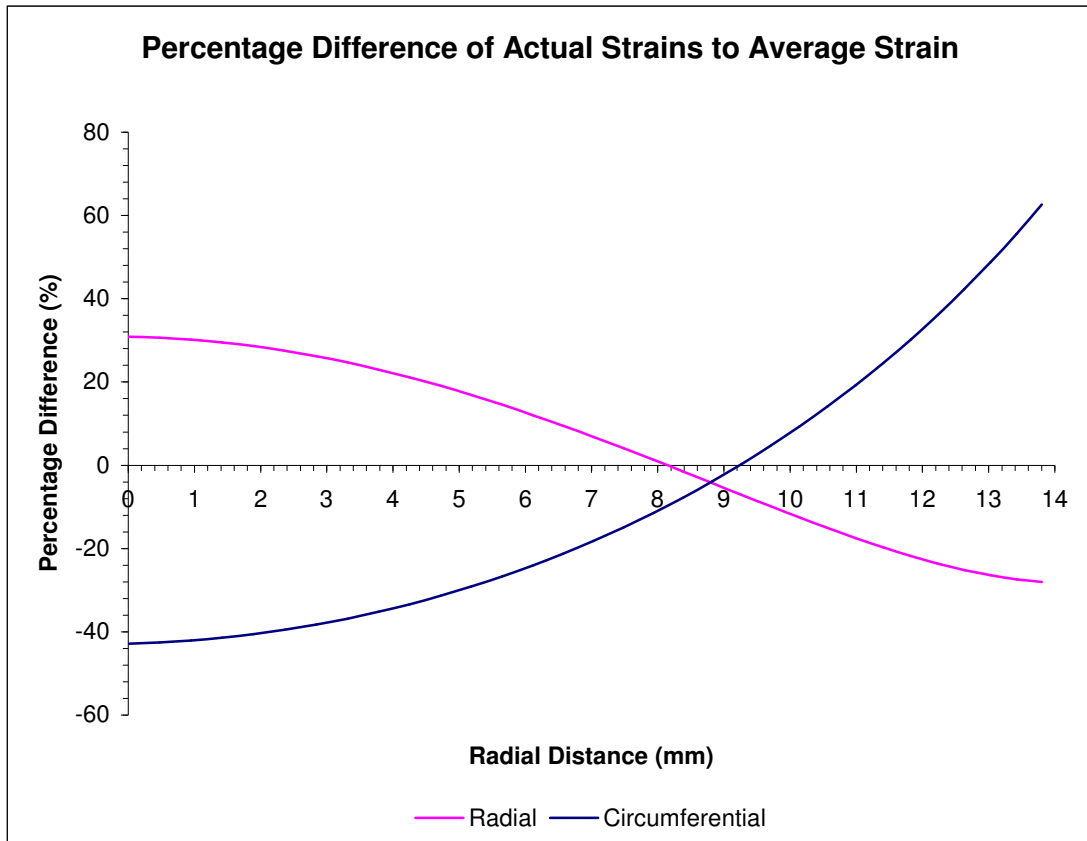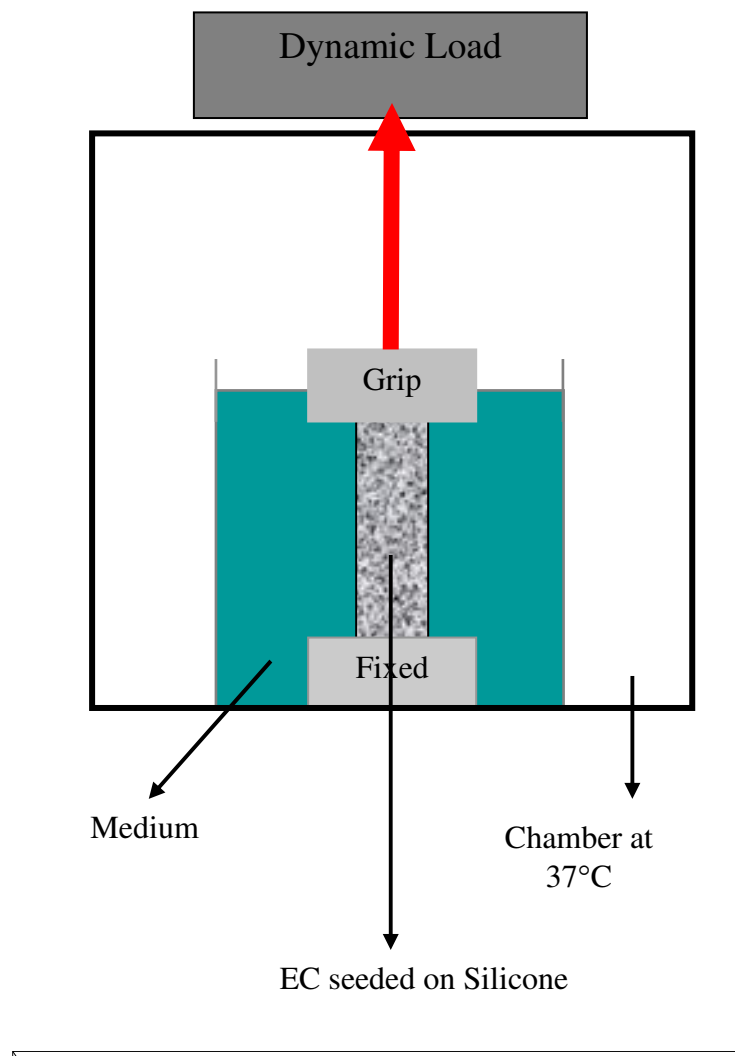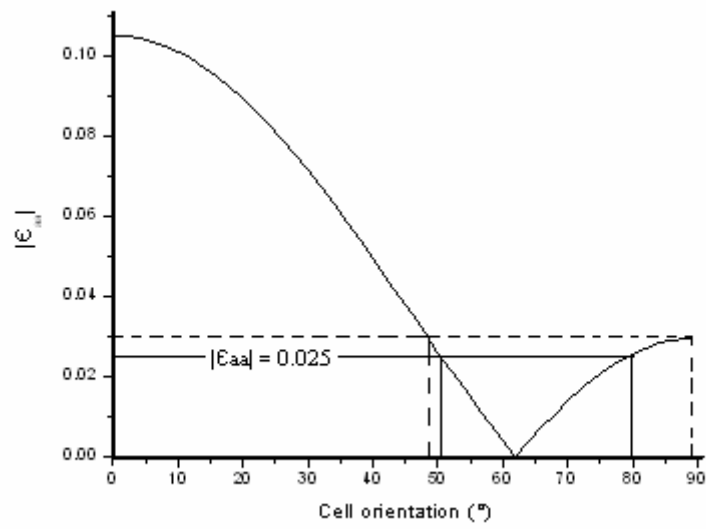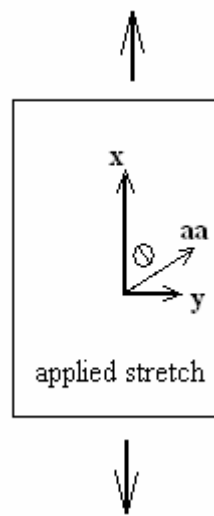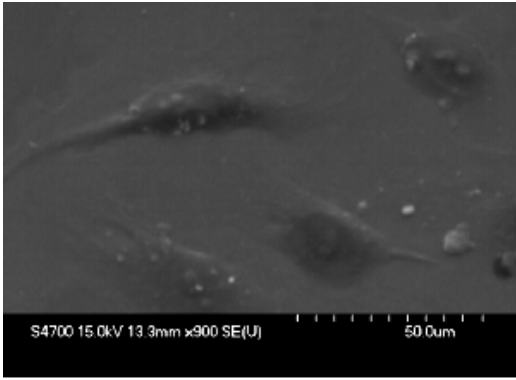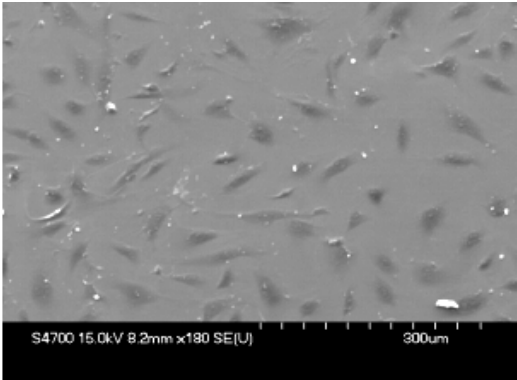
Figure 3 a and b

Figure 4 a and b

Figure 5 a and b
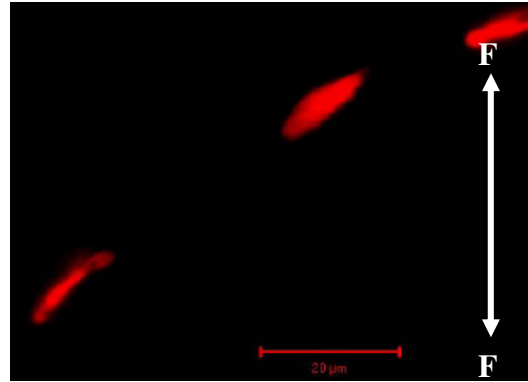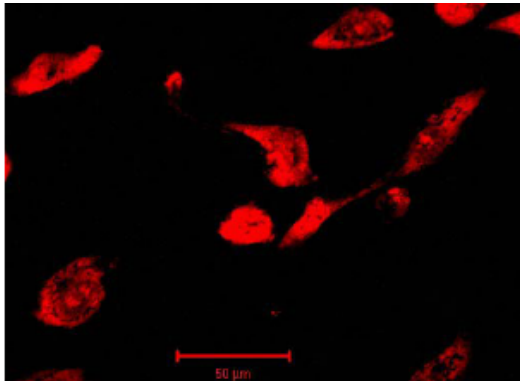
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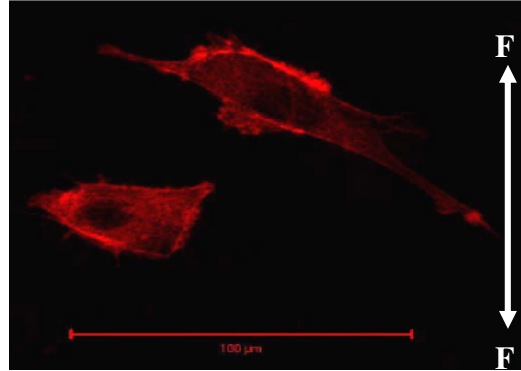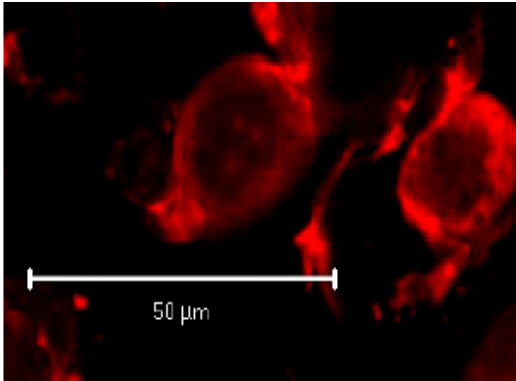
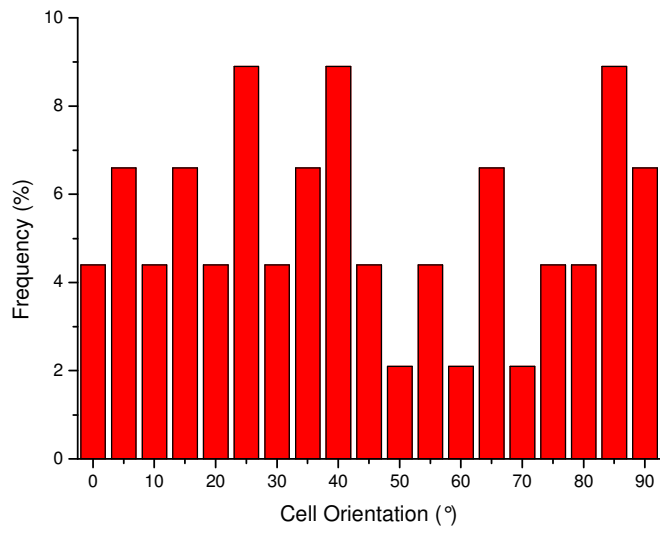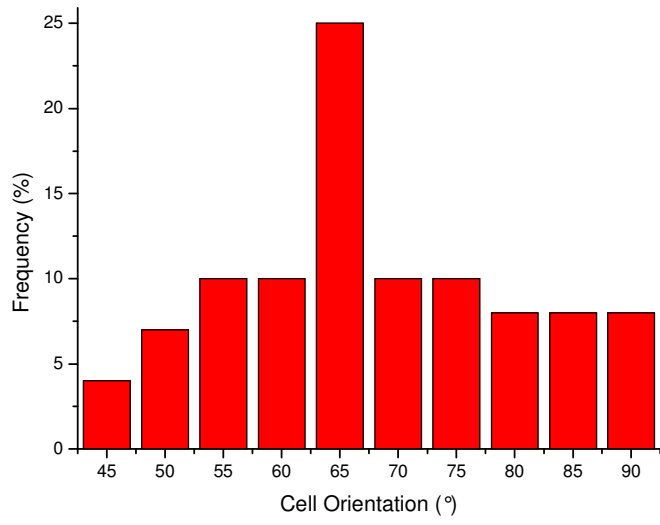Figure 7 a, b, c, and d
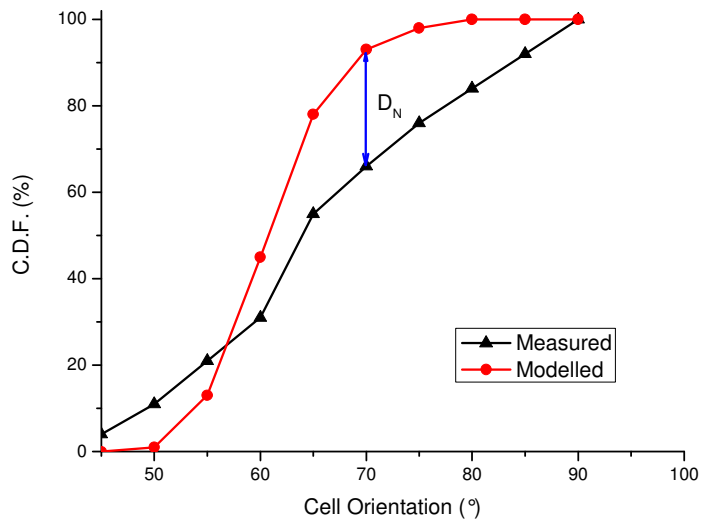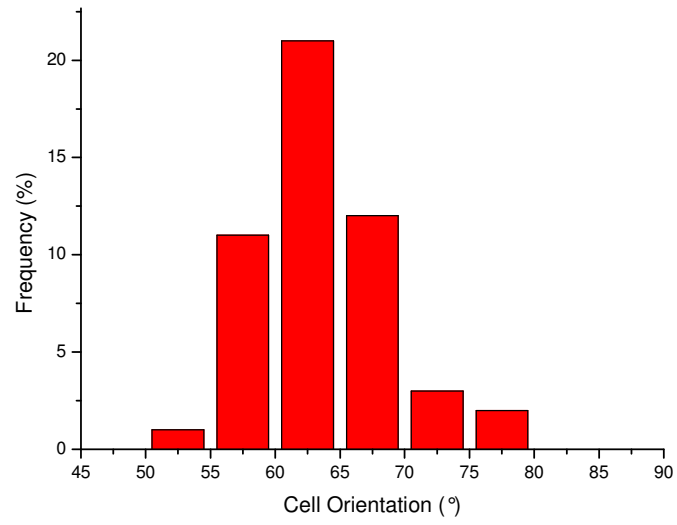
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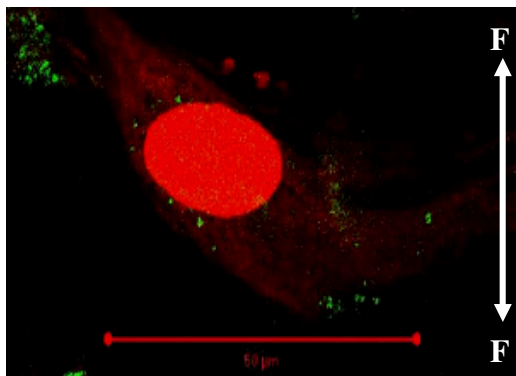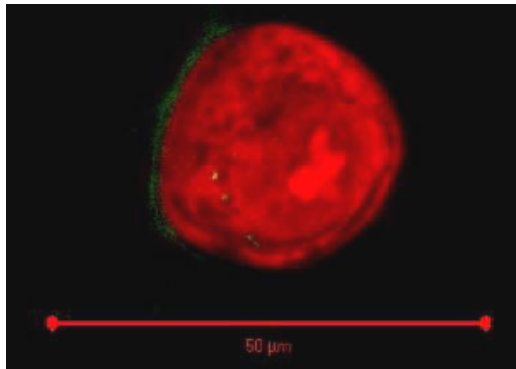
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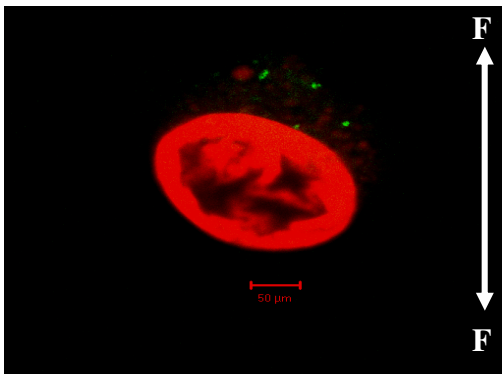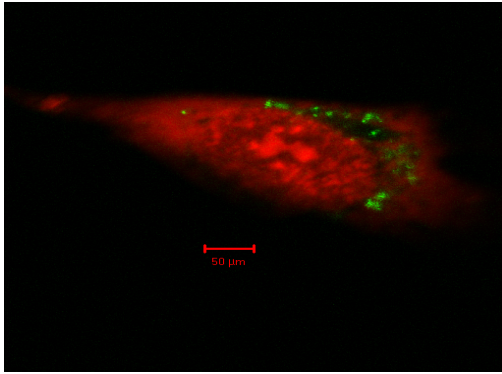
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