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THE EFFECT OF THE VISCOSITY OF THE MEDIUM ON THE REACTIONS OF CELLS TO TOPOGRAPHY

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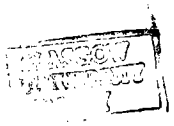
بِسمِ اللّٰهِ الرَحمٰنِ الرَحِيمِ

In the Name of God,
Most Gracious,
Most Compassionate and the Most Merciful



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Dedication

To my Husband Abo-Ulmaali

And

My children Ahmed, Anas and Asmaa

With all my love and grateful

Faten

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ABBREVIATIONS

BHK	Baby hamster kidney
BM	Basement membrane.
BSA	Bovine serum albumin
CC	Close contacts
CMC	Carboxymethylcellulose
C.p	Centipoise
ECM	Extracellular matrix
EC	Extracellular
EDTA	Ethylenediaminetetra acetic acid
EFM	Extracellular fluid macroviscosity
F-actin	Filamentous actin
FA	Focal adhesion
FRET	Fluorescence resonance energy transfer
GAC	Glycosaminoglyocans
Hts	Hematocrits
INA	Illuminating numerical aperture
IRM	Interference Reflection Microscopy
MC	Methyl Cellulose
Mol.wt	Molecular weight
PBS	Phosphate-buffered saline
PLA2	Phospholipase A2
PVP	Polyvinylpyrrolidone
RBC's	Red blood cells
RO	Reverse osmosis
Sb	Standard deviation of the regression coefficient
SiO2	Silicon oxide
TIRFM	Total Internal Reflection Microscope
TTITC	Tetramethylrhodamine isothiocyanate
UV	Ultraviolet

SUMMARY

The viscosity of the medium in which cells grow may alter their cellular reaction to topography and this in turn may affect cell growth, morphology and movement.
In this study, cells have been grown in medium with high viscosity on plane and grooved topography in order to investigate the effect of the viscosity of the medium on the reaction of cells to topography. The viscosity was changed by adding viscous macromolecules, such as dextran, ficoll, polyvinylpyrrolidone, Methylcellulose and Carboxymethylcellulose to the normal medium (ECT or Ham's F10).

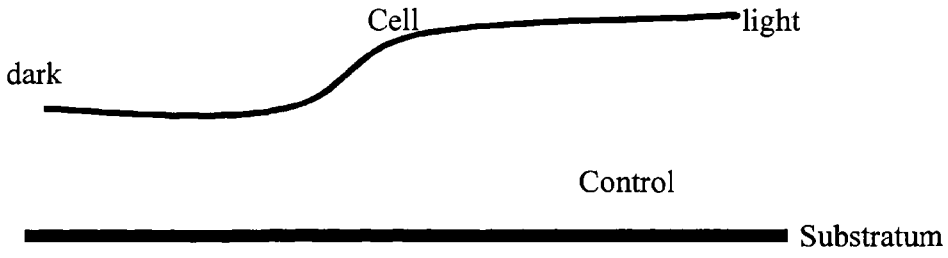
Due to other solution properties that might be altered by the change of medium viscosity, such as osmolarity or chemical interactions, cells were examined in the presence of a variety of concentrations of macromolecules, which differed in their size, capacity to elevate fluid viscosity, and in their chemical nature.
The medium viscosity was determined by measuring its flow time, relative to water, using an Ostwald viscometer. All measurements were performed at 37^0C.
A novel feature of these studies is combining measurement of the medium viscosity with the osmolarity of all media used in the experiments. The osmotic effect could be discounted.

Diffusion was altered when the viscosity of the medium was raised, which in turn affects the reactions of cells to topography. Results from this study have shown that the cell morphology is directly affected when cells are grown in medium of high viscosity. In other words, increasing the medium viscosity decreases the cell length. Increasing the viscosity also reduced cell motility. It also seems that medium viscosity affects the cytoskeleton by limiting its assembly and operation.

Cells were also examined in the medium of high viscosity by ordinary IRM and Live IRM with associated of the refractive index measurements. Results from IRM study show that the space available for diffusion under the cells was decreased when the viscosity of the medium was raised, see the diagram in the next page.

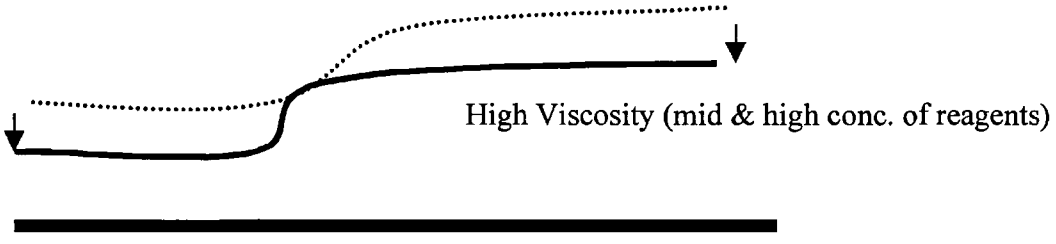
Recovery experiments have shown that were cells still alive in the medium of high viscosity and after they recovered in ordinary medium they spread and aligned again.

The results support the hypothesis that diffusion effects around the cells may be important in the reaction of cells to topography. The results of the present study suggest that elevated medium viscosity is pertinent to biochemical and cellular functions of cells (e.g. plasma and blood cells).



Control

Substratum



High Viscosity (mid & high conc. of reagents)

Chapter One

Introduction

INTRODUCTION

The nature of the interactions of a cell with other cells and with a substrate is an important problem in biology. One method of studying these interactions is to grow cells in tissue culture. When mammalian cells are grown in vitro, most cells do not divide unless they are attached to a solid surface. This surface is commonly covered with an adsorbed layer of serum protein (Penttinen et al, 1958; Saxen and Penttinen, 1965; Giaever and Ward; 1978).

Therefore, it is possible to grow mammalian cells *in vitro* in the laboratory, where the cells act more like independent organisms with a generation time of approximately 24h. For many purposes, it is sufficient, cheaper and more practical to study cells in tissue culture rather than in an intact animal (Carrel, 1912; Giaever and Keese 1986). Early experiments by Harrison (1907), offered reproducible techniques that have been generally accepted as marking the true beginning of tissue culture.

Culture medium is used to maintain the cells. The medium is an aqueous solution of salts, cell growth factors, vitamins, antibiotic agents and possibly serum (Wilkinson et al, 1998).

On other hand, biological cells are also strongly influenced by the topography of the surface on which they live, both in cell culture and in vivo. They are guided along micron sized grooves and change their shape becoming more elongated (Brunette et al, 1983; Clark et al 1990; 1991; Wojciak-Stothard, 1996). These effects can be used for cellular engineering to determine the behaviour of cells and in particular to make prostheses for medical purposes (Wilkinson et al, 1998).

These are important concepts for exploitation in Cell Engineering. According to Wilkinson et al (1998), Cell Engineering can be defined as the use of cells and parts of cell to build systems useful for biomedical purposes and for fundamental research.

TOPOGRAPHICAL REACTIONS

General review

Cells naturally respond to chemical and topographic cues. The response of cells to topography has been recognised for many years.
Many types of cells react to surface topography (Table 1, page 6). One type of such reaction is termed contact guidance. Contact or topographic guidance refers to the reactions of cells to substrate surface morphology and includes alteration in their shape, orientation, adhesion, gene expression, and polarity of their movement (Wood, 1988; Curtis and Clark, 1990). There are at least two components to the reaction, first the alignment of the cells to the substratum and second, the migration of the cells aligns along the substratum features (Wojciak-Stothard et al, 1995). These two components are considered in this thesis.

The reaction of cells to topography occurs during development or in natural tissue regeneration, where extracellular matrix or other cells provide the topographical cues (review of Curtis and Wilkinson, 1997).

History of contact guidance

The effect of topography on cell elongation, cell migration, cell orientation, cell aggregation and cell transformation were reported by numerous authors (Weiss, 1934, 1945; Curtis and Varde, 1964; Dunn and Heath, 1976; Curtis and Clark, 1990; Chehroudi and Brunette, 1995).

Topographical guidance was first observed by Harrison 1910, when he used plasma clots and spider web-fibres in early cell culture experiments. He observed that fibres in the web directed the locomotion and alignment of cells in the cultures.

Weiss in 1934 introduced the term contact guidance when he showed that nerve cells extend their neurites along the fibres in an oriented fibrin clot.
In 1941 Weiss extended his studies by postulating that contact guidance might account for features of morphogenesis. In turn he suggested that the guidance is a reaction to oriented molecules on the fibre surface.
Weiss (1945) then described such effects using nerve cells *in vitro*, when he studied the response of cells and axons to different substrata and locomotion (outgrowth), spreading or migration of the cells and axons in tissue culture. He suggested that cells responded to the molecular orientation of their environment.

Later, in 1964 Curtis and Varde established that the reaction was to the shape of the substrate by growing cells on fibres of varying diameter or grooves, but did not suggest any cellular mechanism. Their observation was that the cells react to features of surface topography when they used diffraction gratings that were V-shaped grooves, that had been ruled by a diamond tool. They also found that highly convex surface of fibres enhanced cell overlap, and grooves diminished cell overlap. They found that the effect of topography on cell behaviour is more probably a direct response by cells to the shape of the substratum. Their observation of cell movement to fibre or groove showed that cells normally move along in straight lines along the fibres. The radius of the fibres was so great that molecular orientation could not be expected. This work was supported by Rovenskey et al in 1971 when they studied cell migration and orientation on grooved substrates, and concluded that cell guidance due to topographical features was a result of differences in the attachment of cells to surfaces with varying geometrical configurations.

Dunn and Heath in 1976 provided further evidence for this theory by proposing that the shape of the substratum imposes mechanical restrictions on the formation of linear bundles of microfilaments, which are involved in cell locomotion, and this determines cell orientation. On the other hand, Ohara and Buck in 1979 limited the directions in which a cell might spread or move, by proposing that focal contacts were aligned in cells and could not form where the cell overlapped a groove edge. But in 1986, Dunn

and Brown showed that the formation of focal contacts in the cell could be on both ridges and grooves, which argues against the theory of Ohara and Buck.

Curtis and Clark in 1990 proposed the idea that sharp discontinuities in the substratum caused actin condensation and that this in turn lead to cell orientation. Oakley and Brunette (1993), used confocal microscopy and grooved substrata to examine the role of microtubules and other cytoskeleton in these reactions. They suggested that microtubule reactions were more important than actin reactions because they were preceded by microtubular orientation.
Later on in 1997, Curtis and Wilkinson reviewed the literature on the reaction of cells to their surrounding topography and summarized the types of prosthesis where advantage could be taken of the ability to fabricate topography.

A more recent theory was proposed in 1998 by Curtis and Wilkinson when they studied the topographical control of cell locomotion and the roles of topographical guidance in cell migration. They postulated that stretch receptors are involved in sensing of topography by cells. Therefore differences in receptors density and degree of anchoring of the receptors to the cytoskeleton could account for differences in the reactions of different cell types to topography.

What is Topography

Topography may be surrounding cells, intracellular materials or biomaterials (Curtis and Wilkinson, 1998). Substratum topography refers to the shape of substrate where cells are growing. Cells appear to be sensitive to surface topography and a wide variety of cell properties can be affected. The surface topographies employed on implanted devices differ markedly not only among implants performing different functions, but also among implants with identical applications (Chehroudi, 1995).

It is also considered that substratum topography (non-cellular components) is an important cue in many developmental processes. Cellular properties such as cytoskeletal organisation, cell adhesion and the interaction with other cells are discussed as being factors determining the susceptibility of a cell to topography (Clark et al, 1990).

Topographic guidance occurs on a variety of natural and synthetic materials. Natural substrata are structures derived from biological materials such as collagen, fibrin and extracellular matrix, which were used in early studies on topography substrata (Weiss, 1945; Dunn, 1982; Singhvi, 1994). The disadvantage of using biological materials is that the chemistry of the material could be affecting the behaviour of the cells and influences the results (Curtis and Clark, 1990).
Materials such as silica and glass (Curtis, 1964; Dunn, 1988), cellulose acetate (Curtis and Varde, 1964), polystyrene (Ohara and Buck, 1979), epoxy resins (Chehroudi et al, 1988), and titania (Brunette et al, 1983) can be used as more controlled substrata.

Surface topographies that produce guidance include grooves with V-shaped, truncated V-shaped (Ohara and Buck, 1979; Dunn, 1982; Brunette, 1986; Brunette, 1988; Chehroudi et al, 1990), rectangular profiles (Clark et al 1990), and hemispherical grooves and ridges (Rovensky and Slavnaya, 1974).
Early methods of fabrication employed lacked flexibility in dimensions and the surfaces were complicated due to heterogeneities introduced during fabrication. Photolithographic techniques solved this problem; feature in the micrometer range can be precisely etched into a variety of substrates.

For an overview of the types of cells, which react to topography and their reactions, see next Tables (1 and 2) on page 6 and 7:

Table 1 Cell types reacting to topography (adapted from Curtis & Wilkinson 1998)

Chondrocytes (D. Hamilton, Thesis 1999)
Endothelia (K. McDonald, Thesis 1998)
Epitena (Wojciak et al., 1995)
Epithelia (Chehroudi et al., 1988 and Clark et al., 1991)
Fibroblasts (Curtis and Varde, 1964)
Leucocytes (Wilkinson et al., 1982)
Lymphocytes (Haston et al., 1982)
Macrophages (Wojciak-Stothard et al., 1996)
Mesenchyme (Wood, 1988)
Neurons (Weiss, 1934, 1945 and Aebischer et al., 1990)
Osteocytes (Vesely et al., 1992)
Oligodendrocytes (Webb et al., 1995)
Smooth muscle cells (Tranquillo et al., 1996)
And some tumour cells (Curtis et al., 1995)
Fungi (Hoch et al., 1987 and Sherwood et al., 1992)

Table 2 Effects on cells of contacting topography (adapted from Curtis and Wilkinson 1998)

Orientation (many studies, especially Clark et al., 1990; 1991)
Align (many studies, e.g. Clark, 1990; 1991)
Normal to topography (Nagata et al., 1993)
Extension enhanced (Curtis et al., 1995)
Accelerated movement (Curtis et al., 1995)
Polarised movement (Dow et al., 1987)
Capture (movement stopped at a particular feature)
Effect on adhesion
Activation of (Wojciak-Stothard, 1996)
Tyrosine phosphorylation (Wojciak-Stothard, 1996)
Actin polymerisation (Wojciak-Stothard, 1996)
Vinculin accumulation over the groove edge (Wojciak-Stothard, 1996)
Phagocytic activity (Wojciak-Stothard, 1996)
Fibronectin m-RNA expression (Chou et al., 1995)

What factors orient cells

Chemotaxis and its role

A cell's environment, to which its movement responds, consists not only of other cells and of a non-cellular substratum, but of a fluid phase. The fluid phase, may influence the direction of a cell's locomotion by chemotaxis (Abercrombie, 1980). Chemotaxis refers to an oriented motile response of a cell or an organism to chemical signals in which movement is affected by the gradient of a diffusible substance, these chemical attract or repel migrating cells (Alberts et al, 1994).

The clearest example of chemotaxis in higher animals is given by neutrophils, the white blood cells that use phagocytosis (reacting to formylmethinonyl peptides) (Zigmond, 1982; Bray, 1992).

It was found that cell behaviour was influenced *in vivo* by chemotactic cues. Chemotactic cues have also been shown to play an important roles in cell guidance in *in vitro* experiments, thus different cell types can detect adhesive cues in their reaction to topography (Clark et al, 1987; Britland et al, 1996).

Chemotaxis also has important roles in the immune response related to cytokines (Abbas et al, 1991). Thus chemotaxis, was believed to be the leading force of directional cell movement for the cells of the immunological system (Wojciak et al, 1996). The chemotactic response has to work over a wide range of concentrations (Bray, 1992).

In addition, it was found that guidance of cells by the substratum surface has some features in common with chemotaxis. Thus, detection of guidance molecules such as fibronectin is made through the specific cell-surface receptors such as integrins. The regions of adhesion are associated with actin filaments as the cell migrates, and may also be sites of local stimulation of actin polymerisation. The range of substances that promote cell substratum migration is probably much larger than those responsible for chemotaxis and there is evidence that specific combinations of extracellular matrix molecules can be recognised. However, at the molecular level the type of response could be quite similar (Bray, 1992).

This is supported by Ingber and Folkman (1987) who state that, studies on cellular mechanisms of signal transduction must focus on the process by which extracellular stimuli alter plasma membrane components and trigger release of intracellular chemical messengers.

Chemokinesis

Chemokinesis, is the phenomenon whereby the cells are stimulated to move more actively by a diffusible molecule in their environments or turn more frequently. Chemokinesis is an interesting and probably developmentally important mechanism, and could well be a crucial factor both in getting invasive movements started, and in keeping them going, whether during normal morphogenesis or the spread of the cancer. But it does not in itself give directionally to moving cells. Incidentally, most chemotactic agents for leukocytes are also chemokinetic. Thus chemotactic messages that give directional information may also promote processes that lead to cell movement itself (Trinkaus, 1982).

Cytoskeleton and cell orientation

The cytoskeleton network contains three principle types of filament: microtubules, microfilaments, and intermediate filaments. Microtubules are highly dynamic structures that alternately grow and shrink by the addition or loss of tubulin subunits. Actin filaments are also dynamic structures but they normally exist in bundles or networks. A layer called the cortex is formed just beneath the plasma membrane in many cells from actin filaments and a variety of actin-binding proteins. This actin-rich layer controls the shape and surface movement of most animal cells. Intermediate filaments are relatively tough, ropelike structures that provide mechanical stability to cell and tissues. The three types of filaments are connected to one another and their functions are coordinated (Bray, 1992).

Current hypotheses on the mechanism of contact guidance focus on the dynamic behaviour of the cytoskeletal components (Wojciak-Stothard et al, 1995).
The contact of cells with patterned substrata induces several changes in cell morphology and activity and is accompanied by changes in the distribution and contact of cytoskeleton components (Wojciak et al, 1996). Curtis and Britland (1996), suggested that the cytoskeleton was affected by surface topography, which in turn affects cell shape. As mentioned before Oakley and Brunette in 1993 were the first to examine the role of microtubules and other cytoskeletal elements in the reaction of cells to topography. Wojciak et al, 1995 concluded that the aggregation of actin along groove/ridge boundaries is a primary driving event in determining fibroblast orientation on microgrooved substrata.

Ingber and Folkman concluded from experimental observation, that the cell may be considered as a tensegrity system since it is comprised of a discontinuous arrays of a compression-resistant structures (e.g., microtubules) that are interconnected by a continuous series of tensile elements (e.g., contractile microfilaments).
In epithelial cells, according to the Ingber hypothesis (1987), of elastic tensegrity structures, the basement membrane (BM) should regulate cell shape as well as growth and differentiation through its transmission of the physical forces of tension and compression for a given spatial configuration. In this manner, the structural coordination, homogeneity of cell form, and effective systems of intercellular communication required for successful tissue function are sustained by normally constant architectural relationships.
ECM-dependent alterations of cell geometry that affect cytoskeleton polymerisation could alter cell responsiveness to soluble mitogens by a variety of mechanisms. The cytoskeleton appears to serve as an intracellular orienting scaffold that can control the position, and possibly the function, of much of the cell's metabolic machinery. For example, effects of cell shape on cell metabolism could be mediated by associated alterations of membrane transport proteins or growth factor receptors since the distribution, motility, and function of plasma membrane proteins may by regulated by the cytoskeleton (Ingber and Folkman, 1987).

Attachment and spreading of cells on a surface involves the organisation of microtubules, microfilaments and intermediate filaments (Abercrombie and Dunn, 1975).

Structure and organisation of actin filaments

The major cytoskeletal protein of most cells is actin, which polymerizes to form thin filaments, which are approximately 7 nm in diameter and up to several micrometers in length. Within the cell, actin filaments are organised into higher-order structures, forming bundles or three-dimensional networks with the properties of semisolid gels. The assembly and disassembly of actin filaments, their cross-linking into bundles and networks, and their association with the other cell structures (such as the plasma membrane) are regulated by a variety of actin-binding proteins, which are critical components of the actin units. In particular, the actin-binding domains of many of these proteins are similar in structure. They are separated by spacer sequences that vary in length and flexibility, and it is these differences in the spacer sequences that are responsible for the distinct cross-linking properties of different actin-binding proteins, Figure 1 (Cooper, 1997).

Protrusions of the cell surface

The surface of most cells has a variety of protrusions or extensions that are involved in cell movement, phagocytosis, or specialised functions such as absorption of nutrients. Most of these cell surface extensions are based on actin filaments, which are organised into either relatively permanent or rapidly rearranging bundles or networks (Cooper, 1997).

Many cells also extend thin projections of the plasma membrane supported by actin bundles. The formation and retraction of these structures is based on the regulated assembly and disassembly of actin filaments (Cooper, 1997).

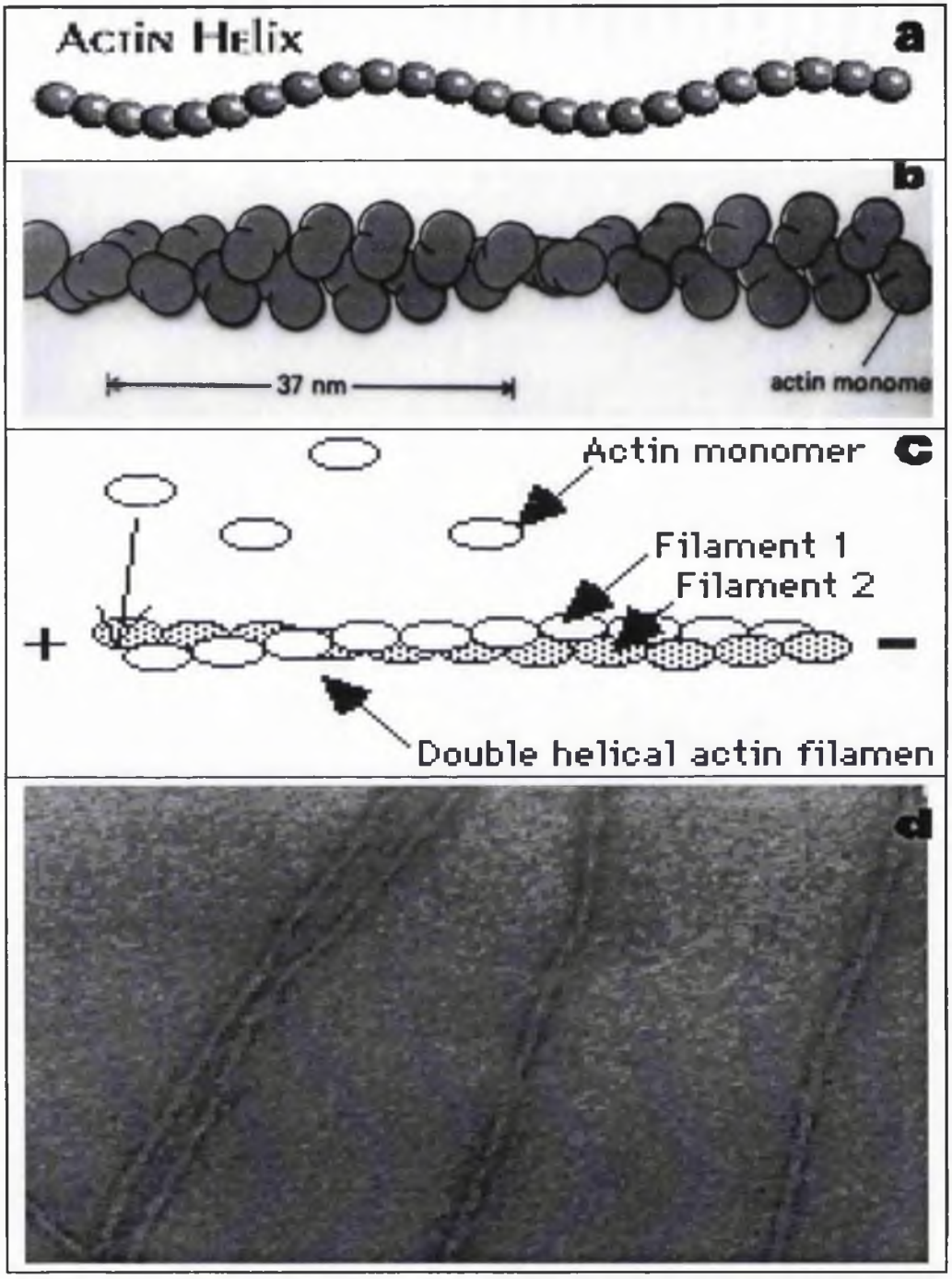


Figure 1

Actin thin filament structures: (a) actin helix, (b) double helical actin filament, (c) structures of actin filaments, and (d) An electron image of actin filaments. (adapted from web sites 1 & 2).

Cell properties affected by surface topography

Chehroudi and Brunette (1995) reviewed the previous studies (Weiss, 1945; Curtis and Varde, 1964; Dunn and Heath, 1976; Curtis and Clark, 1990) about topography that had been employed in biomaterials or cell biological research, and the processes involved.

They concluded in their review that the surface topography of an implant is well established as an important factor affecting several cellular functions (cell shape, migration, adhesion, and tissue organization). There is a good deal of information on how the surface topography of an implant can affect the morphology of adjacent cells and tissues.

In general according to Chehroudi and Brunette (1995) these papers identify the following effects:

Cell adhesion: Surface topography can affect cell adhesion, but the particular effect depends on cell type.

Orientation and contact guidance: Contact guidance (as mentioned before) refers to the tendency of cells to be guided in their direction of locomotion by the shape of the substratum. Topographic guidance can occur on artificial substrata *in vivo* and perhaps in the body.

Tissue organization: The surface topography of an implant can influence the orientation and the organisation of adjacent tissues. Cell traction can result in tissue organisation, in particular, into ligament-like collagen-containing structures. Tissue alignment *in vivo* can also be affected by topography (Chehroudi, 1988).

Cell polarity: Cells migrating on a substratum may exhibit structural polarity such as the fan-like shape of migrating fibroblasts and persistent movement in out direction. The implants that limit diffusion of materials to cell might well alter cell polarity and function.

Differentiation: *in vivo* differentiation often occurs as a result of specific cell interactions, with a defined spatial relationship between the interacting cell populations. It is possible that substratum topography as well as substratum chemistry could be modified to produce desired cell functions.

Cell shape: The surface topography of many implanted devices can alter cell shape, see Table 2.

Cell adhesion

Cell adhesion is a crucial for assembly of individual cells into the three-dimensional tissues of animals. A variety of cell adhesion mechanisms are responsible for assembling cells with their connections to the internal cytoskeleton, determining the overall architecture of the tissue.

Barry, 1996 reviewed the functional units of cell adhesion as typically multiprotein complexes made up of three general classes of protein; the cell adhesion molecules/adhesion receptors, the extracellular matrix (ECM) proteins, and the cytoplasmic plaque/peripheral membrane proteins. The cell adhesion receptors are usually transmembrane glycoproteins that mediate binding interactions at the extracellular (EC) surface and determine the specificity of cell-cell and cell-ECM recognition. They include members of the integrin, cadherin, immunoglobulin, selectin, and proteoglycan superfamilies. At the EC surface, the cell adhesion receptors recognize and interact with either other cell adhesion receptors on neighbouring cells or with proteins of the ECM. ECM proteins are large glycoproteins including the collagens, fibronectins, laminins, and proteoglycans that assemble into fibrils or other complex macromolecular arrays.

At the intracellular surface of the plasma membrane, cell adhesion receptors associate with cytoplasmic plaque or peripheral membrane proteins. Cytoplasmic plaque proteins serve to link the adhesion systems to the cytoskeleton to regulate the functions of the adhesion molecules, and to transduce signals initiated at the cell surface by the adhesion receptors.

The adhesive interactions between a cell and its surrounding ECM regulate its morphology, migration, growth, and differentiation (Burridge and Wodnika, 1996). It was shown that surface topography could affect cell adhesion (Curtis and Clark, 1990; Chehroudi and Brunette, 1995; Curtis and Wilkinson, 1998).

Focal adhesions (FA)

One system for adhesion to ECM is the focal adhesion (FA).

Focal adhesions are sites of tight adhesion to the underlying extracellular matrix developed by cells in culture and characterised by narrow gaps (10-20nm) between the lipid bilayer of the cell membrane and the glass surface at the focal contact. They provide a structural link between the actin cytoskeleton and the extracellular matrix and are regions of signal transduction that relate to growth control (Abercrombie et al, 1971; Brunk et al., 1971; Harris, 1973; Abercrombie and Dunn, 1975; Begg et al., 1978; Heath and Dunn, 1978; Bereiter-Hahn et al., 1979; Goldman et al., 1979; Izzard and Lochner, 1976, 1980; Chen, 1981; Burridge and Wodnika, 1996).

Transmembrane components in FAs are integrins, a large family of transmembrane heterodimers (Hynes, 1992).

Close contacts (CC)

Close contacts are broader areas often found surrounding FA sites, which are characterised by larger gap (30-50nm) between the cell surface and substratum. A meshwork of microfilaments is often seen subtending the membrane at these sites (Izzard and Lochner, 1976, 1980; Heath and Dunn, 1978; Couchman and Ress, 1979; Bereiter-Hahan et al., 1979).

Extracellular matrix (ECM) contacts

Extracellular matrix contacts are sites where strands and cables of extracellular matrix materials including fibronectin connect the ventral cell surface to the substratum where they are separated by large distances (>100nm) (Chen et al., 1978; Hedman et al., 1978; Singer, 1979; Furcht et al., 1980).

Cell shape

There is evidence that surface topography can alter cell shape (Weiss, 1945; Brunette, 1988; Clark, 1989; Cherhroudi et al, 1995).

Two amongst the main effects that appear to control cell behaviour and morphology in tissue culture are, according to Curtis et al, 1964, the effect of population density on both overlap and cell spreading (Abercrombie, 1975), and secondly the effect of the topography on overlap behaviour, population density and cell spreading.

Since there have been many suggestions that topographical reactions of cells may play a role in embryogenesis, regeneration and wound healing, it is thought that topography can affect cell morphology and cell behaviour (Bard and Higginson, 1977; Newgreen, 1989; Wylie et al, 1979; Webb et al, 1995; Curtis and Clark, 1990).

Clark (1994) studied the role of the local environment in influencing the behaviour of cells and it was found that topography of a substratum could dramatically alter cell shape and guidance. This was dependent on number of factors includes step height or groove depth, the density of the cue, cell type and cell density.

Cell locomotion

It is known that the locomotion of animal cells involves four steps: protrusion of a lamellipodium, pseudopodium, or other locomotor extension at the cell front; adhesion of this extension to the substratum; and retraction of the cell body toward the front, and detachment of the rear part of the cell from the substratum (Elson et al, 1998), see Figure 2.

However, it is not necessary that these steps occur separately, moreover the cell could exert contractile forces on the substratum while it extends forward (Harris, 1994).

In each step, the cell exerts force on itself to change its shape and/or on the substratum to detach or to drive itself forward (Elson et al, 1998), (see Cellular Mechanical Properties). Therefore directed cell movement by topography is a phenomenon recognised as being crucial in the development of multicellular organisms (Dunn et al., 1976 and Clark et al, 1987).

It also been observed that cells move with higher speed on patterned substrata than on plane substrata (Wojciak et al, 1995).

Cells not only look different on surfaces of differing topography, but also function differently as illustrated by Clark et al., 1990 and Chehroudi et al., 1995.

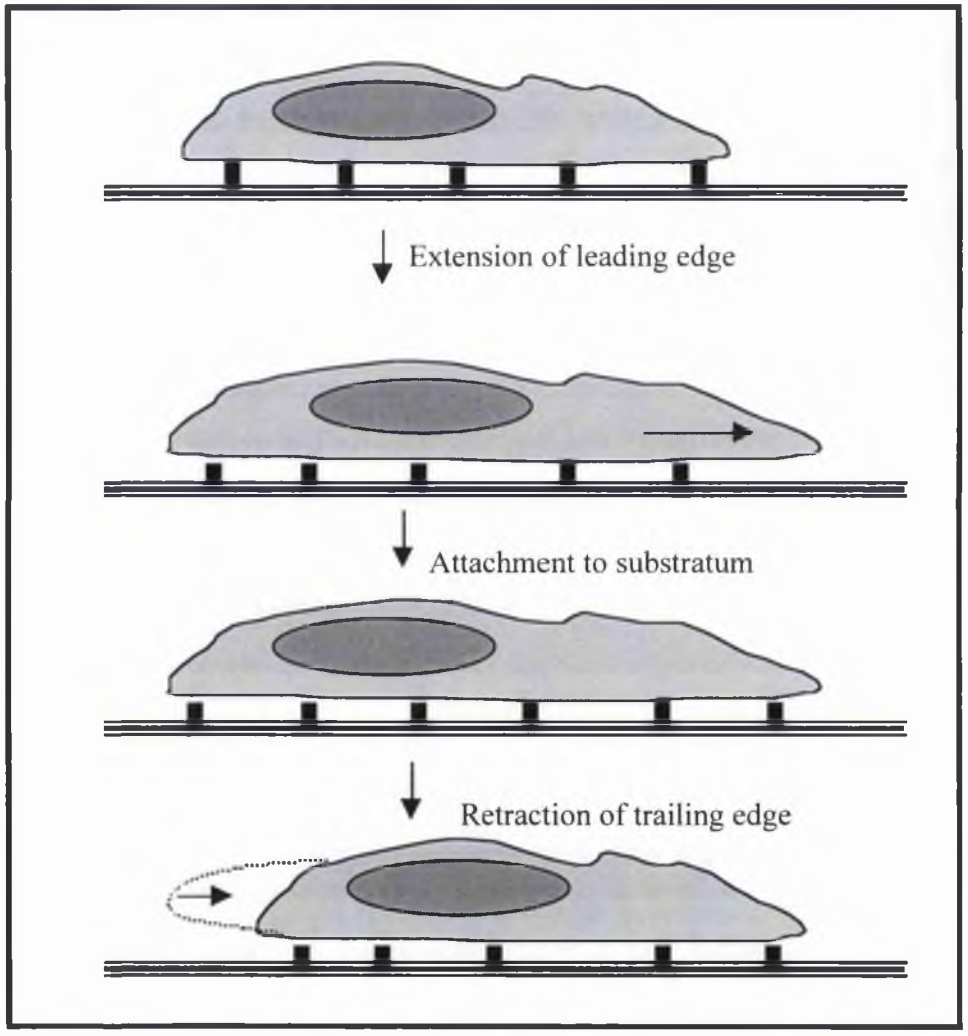


Figure 2

The movements of cells across a surface (adapted from Cooper, 1997).

London-van der Waals' and Electrostatic forces

Intercellular and cell-to-substratum adhesion are related to two main forces, electrostatic forces, which are repulsive between similarly charged surfaces, and London-van der Waals' (electrodynamic) forces, which cause attraction between similar bodies (Gingell and Vince, 1979). Gingel and Vince (1979) have shown that in certain situations cell contact can be dependent on electrostatic repulsion.
The major part of the difficulty of accounting for cell adhesion in physical terms almost certainly lies in the extreme complexity of the cell surface and, in particular, its specialised contact regions; it is inevitable that the charged components of the surface which generate electrostatic fields play some part in contact processes; likewise it is certain that electrodynamic forces act between cell surfaces as they come into apposition.
If most adhesive contacts are molecular, it would not be surprising to find correlations between surface energy and adhesion (Gingell and Vince, 1979).
Long-range van der Waals attraction and long-range electrostatic repulsion, which obey different force laws, can balance at a finite separation (secondary minimum) and at the limit of close approach (molecular contact) which is a more energetically stable position (Parsegian and Gingell, 1973).

Van der Waals' attraction

Electrodynamic forces arise between atoms, molecules and assemblies of molecules due to their vibrations, giving rise to electromagnetic interactions; these are attractive when the vibrational frequencies and absorptions are identical or similar, repulsive when non-identical. Other interactions originally proposed by van der Waals were included in this name, but these are usually separated into Coulomb force, the Keesom force and the London force. Only the last is of electrodynamic nature, probably important in holding lipid membranes into the structure and possibly in other interactions, e.g. cell adhesion. Electrodynamic forces between large-scale assemblies can be relatively long-range nature (Lackie and Dow, 1995).

Long range forces of attraction were originally detected by Derjaguin et al. (1956) and the many subsequent measurements have been reviewed by many authors (Israelachvili, 1974; Israelachvili, and Adams, 1978; Parsegian, 1975; Shih and Parsegian, 1975). Such forces, capable of operating over relatively large distances (tens of nanometers) in physiological media, were first postulated to be important in adhesion by (Bangham and Pethica, 1960; Curtis, 1960, 1973).

Short-range forces

Short-rang forces, such as steric repulsion, hydration forces, hydrogen-bonding etc., may also come to play at very small separations; in this regime the distinction between chemical and physical forces becomes semantic. Antigen-antibody interaction, for example, ultimately depends on electrostatic and electrodynamic forces acting over small distances, complementary interactions by hydrogen-bonding; the overall interaction energy depends on optimal juxta-position of interacting atoms, which is dictated by steric factors (Gingell and Vince, 1979).

Increasing the cell surface charge density (and hence the electrostatic repulsion) prevents cell adhesion and causes detachment of already adherent cells (Gingell and Vince, 1979).

CELLULAR MECHANICAL PROPERTIES

Cells respond to physical factors and chemical factors, often through the same cytoplasmic machinery. Mechanical stress, in particular, has a major influence on cell movements (Bray, 1992).

One mechanism by which externally applied mechanical force might change the cell is by acting directly on the cytoskeleton (Bray, 1992). In smooth muscle and non-muscle cells, one of the regulatory system of the cytoplasm involves phosphorylation of the myosin regulatory light chains (Burridge and Wodnika, 1996).

Cellular mechanical properties are directly derived from measurements of cellular deformability, self generated or applied. Generally it would be desirable to have quantitative estimates of the elasticity and viscosity moduli for the cell in different physiological states and while participating in various interactions with other cells and substrates. Measurements on mechanically or functionally distinct regions of individual cells would also be more informative (Elson et al, 1998).

Cell locomotion as mechanical process

Cell locomotion is essentially a mechanical process. To translocate, cells exert forces both on themselves to change shape and on the substratum to which they are attached to drive themselves forward. The forces that resist cell-shape change arise from passive cellular viscoelasticity, active regulatory mechanisms, and interaction of cells with other cells as well as substratum they are attached to. Resistance to translocation arises mainly from adhesive interaction with the substratum. Cell shape and translocation are governed by the balance of these opposing forces (Elson et al, 1998).

Protrusion is the pushing forwards of parts of the membrane. A distinction is commonly made between traction and contraction forces in cellular locomotion (Harris, 1994) in terms of the location of the effect. Traction describes the forces that pull a cell forward toward its leading edge and drive it to spread over a substratum.

Contraction has the opposite effect of drawing the cell or some portion of it into a compact shape. Therefore myosins are likely to generate both types of forces (Elson et al, 1998).

Observations of cellular processes driven by forces should be linked to locomotion (systematic transport of surface particles), measurements of the effective changes in conditions (strength of adhesion to the substratum and the presence of chemotactic agents) (Elson et al, 1998).

A method was developed to measure forces exerted by a variety of adherent cell types by Harris et al, 1980. The forces exerted by cells on a flexible silicone rubber substratum were characterised in terms of the wrinkling of the rubber layer. The importance of this work was the demonstration that different cell types vary in the strength of the forces they exert on the substratum, and that there is an inverse relationship between their velocity of translocation and the strength of these forces.

Viscoelastic mechanical properties

Initial studies by Petersen demonstrated that the measured deformability of cells includes both elastic and viscous components, the latter of which causes the measured cell stiffness to increase with rate of indentation, other matters being equal. This means that cells become softer, and the viscous contribution less as the temperature is increased from 30^{0} C to 37^{0} C. The disruption of actin microfilaments by cytochalasins causes a cell to become substantially softer, and the deformability varies from place to place on the cell (Petersen et al, 1982).

Viscoelasticity plays an important role in function of cells. It is a key factor in the regulation of cell shape at resting, and in moving cells (Bausch et al, 1998). Furthermore, Sato et al. (1996) suggested that viscoelastic properties are considered to be closely related to cytoskeletal structures.

Bausch et al, 1998 stated the cell viscoelasticity is determined in a complex way by the composite shell envelope composed of the lipid-protein bilayer with the associated actin cortex and the internal cytoskeleton composed of actin

microfilaments, microtubules, intermediate filaments, and their associated proteins. High precision measurements of viscoelasticity parameters of cells are thus expected to give insight into the structure of the cortical and internal cytoskeleton and to quantify the effect of drugs, mutations, or diseases on the cell structure.
Viscoelasicity measurement must include three conditions: allow local measurements on micrometer to nanometer scales, allow repeated measurements, and the data analysis should be independent of a specific cell model.
Viscoelastic behaviour of the cell in contact with substratum is characterised by three parameters: an elastic constant, a relaxation time, and viscosity (Bausch et al, 1998).

Measurements of adhesion

In principle there are two approaches to measuring quantitatively cell adhesion: physical distraction of a preformed adhesion or monitoring of the rate of cell-cell or cell-substrate collision. The approach of pulling apart an adhesion suffers from many difficulties with interpretation of data because the cells often fracture internally. The second approach has therefore been extensively used. In collision studies, isolated cells are brought into contact by the flow of the suspending medium which is agitated in some manner. A collision would in most cases result in a reseparation since the medium flow would still be acting, but if there is an adhesive interaction between the colliding cells which is strong enough to withstand the shearing forces of the medium, then an aggregate will be formed. The most widely used method for assessing the adhesiveness of the cells is that in which the time course of aggregate formation is monitored (Curtis and Lackie 1991).
Adhesion can also probably be characterised qualitatively by measuring cell spreading or the presence of focal contacts. Other quantitative measurements of adhesion involve application of a known force to a cell in order to detach the cell. Adhesion strength can be measured using any of a number of techniques which permit the application of a known force on a cell including micropipette aspiration, hydrodynamic flow and laser tweezers.

In addition to the above measurements, determination of the cell -substrate contact area permits calculation of the bond forces involved in adhesion and the mode detachment Curtis and Lackie (1991). Contact areas and cell-substrate separation distances can be measured by electron microscopy, interference reflection microscopy and related variations, and total internal reflection fluorescence microscopy Burmeister (1998).
In bovine aortic endothelial cells (BAEC), cell adhesion increases with increasing contact area, but for contact area greater than $65\mu m^2$ per cell further increases in contact area had little effect on the strength of cell adhesion Burmeister (1998).

Another study suggested that cell deformability is dependent on cell shape and extent of adhesion to substratum, fibroblasts have a rounded shape when they adhere poorly to a substratum spread well on an adhesive substratum (Elson, 1988).

Mechanical properties of the cells and medium viscosity

The medium viscosity might affect the magnitude of the forces producing collision or distraction (tearing out), for the adhering cells (Curtis and Lackie, 1991). There are a number of assumptions that have to be made when dealing with a fluid in which the viscosity is a function of shear stress. The shape of the particle will affect the way in which it moves through the flow /or flows through a tube. The geometry of the collisions (for the adhering systems) will be different if a long thin particle collides end-on or side-on. The deformability of the particle will determine how large an area comes into contact with the substratum, and the plasticity of the particle may then cause it to respond to fluid flow by elongation passively, thereby reducing the distraction force. Most cells are probably too rigid to deform much upon collision or to be passively deformed following attachment; shape changes in cells are usually active processes. Therefore the filopodia of the cells (protrusion from the cell surface) may readily be deformed by the collision (Lackie, 1991).
Curtis and Lackie, 1991 concluded that viscosity comes into a collision situation twice. First to determine how frequent collision will occur and secondly in

determining how rapidly and powerfully particles will be thrown together and then dragged apart during collision.

Particle distribution

Molecules are normally distributed or spread more or less randomly over the cell surface. The presence of large molecules in the medium such as dextran can shield charge on the cell surface and allow aggregates to form as in isolating Leucocytes from blood cells by adding dextran (Curtis and Lackie, 1991).

It is most important to note that viscosity influences all molecular motion including diffusion of substrates and products. In other word increased viscosity slows diffusion of molecules in and/or out of cells.

PHYSICAL RELEVANT FACTORS

Diffusion between cells

In general, matter diffuses from a region of higher to lower concentration. In inquiring how molecules move it is instructive to review a property of the free diffusion of natural molecules.

In his random-walk model Einstein (1908) showed that free diffusion and Fick's law are accounted for by the random thermal agitation of individual molecules.

A molecule does not need to 'know' what its neighbors are doing, it does not need to know of the existence of a gradient; by moving randomly to the right and to the left with equal probability biased by concentration differences it will make its contribution to the diffusion flux (Hille, 1971).

Passive diffusion

A cell in suspension moves passively due to diffusion though some can swim (Bray, 1992), molecules around the cell do the same. The velocity of diffusion per unit force so that Flux = Concentration x Mobility x Force.

It is usual to define the forces producing these fluxes as the negative gradients of free energy. The most common free energy gradients encountered are the gradient in chemical potential in the case of diffusion of a natural particle, and the gradients in electrochemical potential in the case of diffusion of charged particles in the presence of an electric field.

In the case of free diffusion of a neutral molecule, the gradient in potential energy is equal to the gradient in chemical potential, which is said to be "Fickian". Conversely, any departure from Fick's law for neutral molecules is often taken to indicate non-passive diffusion processes. The case of Fickian diffusion can be exploited further to define some additional useful terms and an important theoretical problem (Sjodin 1971).

The diffusion coefficient (D_i) according to diffusion Fick's law is $D_i = U_i RT$

the proportionality coefficient U_i is the mobility of species I, R is the gas constant and T is the absolute temperature.

Molecules in solutions move in a random fashion due to the continual buffeting that they receive in collisions with other molecules. This movement causes molecules to diffuse over intracellular distances in a surprising short time (Alberts et al., 1994).

Diffusion in the extracellular space

Extracellular space is filled by an intricate network of macromolecules constituting the extracellular matrix. This matrix is composed of a variety of versatile proteins and polysaccharides that are secreted locally and assembled into an organised meshwork in close association with the surface of the cell that produced them (Alberts et al., 1994). In between lies the tissue fluid often called the tissue utrafiltrate.

The matrix plays an active and complex role in regulating the behaviour of the cells that contact it, influencing their development, migration, proliferation, shape, and

function. The extracellular matrix has a correspondingly complex molecular composition (Alberts et al., 1994).

The macromolecules increase the viscosity of the extracellular space Curtis (personal communication).

The macromolecules that constitute the extracellular matrix are mainly produced locally by cells in the matrix. In most connective tissues the matrix macromolecules are secreted largely by fibroblasts. The two main classes of extracellular macromolecules that make up the matrix are 1. polysaccharide chains of glycosaminoglycans (GAC), which usually linked to proteins to form proteoglyocans, and 2. fibrous proteins of two functional type: mainly structural (e.g. collagen and elastin) and mainly adhesive (e.g. fibronectin and laminin). The polysaccharide resists compressive forces on the matrix, and the collagen fibres provide tensile strength.

The aqueous phase of the polysaccharide gel permits the diffusion of nutrients, metabolites, and hormones between the blood and the tissue cells (Alberts et al., 1994).

The polysaccharide chains fill most of the extracellular spaces, providing mechanical support to tissues while still allowing the rapid diffusion of water-soluble molecules and the migration of cells (Alberts et al., 1994). This is because of the extended nature of GAC chains, and their hydrophilic properties, which can restrict the movement of large molecules through the matrix but allow relatively free diffusion of small molecules (Boubriak et al., 2000).

The diffusion displacement distances are comparable with cellular dimensions, raising the possibility that the measurements of water diffusion might provide a means of exploring cellular integrity and pathology (Eriksson et al., 2001). Tissues with more hindered diffusion in one direction than another are said to be anisotropic whereas diffusion is the same in all directions in isotropic tissues.

Pathological processes that change the microstructural environment, increased or decreased extracellular space and loss of tissue organisation, result in altered diffusion and/or anisotropy. Increased diffusivity could be caused by a cell loss resulting in increased extracellular space (Eriksson et al., 2001).

Diffusional transport of solutes through matrix can be characterised by diffusion and partition coefficients (Marouds and Urban, 1983).

Boubriak et al., 2000 studied the effect of matrix composition on solute diffusion in rabbit sclera, they have found that (1) diffusion and partition coefficients are sensitive to solute molecular weight, decreasing as MW increases; (2) diffusion and partition coefficients are sensitive to tissue hydration, increasing as hydration increases; and (3) removal of glycosaminoglycans has only a small effect on either diffusion or partition coefficient.

Osmotic pressure

The phenomenon of osmosis (from the Greek word for 'push') is the spontaneous passage of a pure solvent into a solution separated from it by a semipermeable membrane, a membrane permeable to the solvent but not to the solute. The osmotic pressure is the pressure that must be applied to the solution to stop the influx of solvent. One of the most important examples of osmosis is transport of fluid through cell membranes, but it is also the basis of osmometry, the determination of molar mass by the measurement of osmotic pressure. Osmometry, is widely used to determine the molar masses of macromolecules (Weast, 1975; Atkins, 1998). Intracellular volume changes depend strongly on solvent diffusion and therefore, on osmotic phenomena. This is the pressure resulting in an ideal solution due to the presence of impermeant solute in it.

Many cellular membranes are leaky to probing molecules. Solute moves between two compartments separated by a membrane and solvent diffuses until the system finally reaches equilibrium in which the two compartments are identical in pressure and composition (Schwartz, 1971).

Viscosity

Liquids exhibit resistance to flow known as viscosity. In general, it is the property of resistance which opposes the relative motion of the adjacent portions of the liquid and can consequently be regarded as a type of internal friction (Glasstone, 1948).
Bryant Chase, 1998 suggested that increased viscosity of a solvent decreases chemical reaction kinetics.
The movement of an object under a given force is slowed as viscosity increases. Thus the movement of a cell through intercellular space may be retarded by high viscosity media. Are effects of high viscosity media due to this or to effects on diffusion?

The coefficient of viscosity

The coefficient of viscosity η is defined as the force per unit area, dynes-seconds per sq. cm.(dyne-sec/cm^2), required to maintain unit differences of velocity, i.e., 1 cm. per sec. (cm/sec), between two parallel layers 1 cm. apart (Glasstone, 1948). In order to maintain a uniform velocity a steady force must, therefore, be applied to overcome the influence of the viscosity of the liquid. It has been found (Stokes, 1850) that if a small sphere of a radius r travels at a velocity u through a fluid, gas or liquid, having a coefficient of a viscosity η, the force applied which just balances that viscosity, is given by Stokes's law

$$f = 6\pi r\eta u$$

Centipoise (Cp) = 0.01 poise (unit of absolute viscosity = gm/sec x cm).

Measurements of viscosity

One method for the direct measurement of viscosity requires the determination of the rate of flow of liquid through a capillary tube of known dimension (Glasstone, 1948). The method commonly employed involves an instrument known as a viscometer of which several types are available, (see Materials and Methods in the next chapter).

Variation of medium viscosity

Fluid inhibiting internal friction (transport of momentum) is said to be viscous. The viscosity of medium is modified in many studies by the addition of various macromolecules to the medium (Hovav et al, 1987; Yedgar and Reisfeld, 1990; Tuvia et al, 1997; Armitage and Packer, 1998).

Although variation of solvent viscosity is a useful probe applied in studies of protein dynamics, enzyme function, effect of plasma viscosity on blood cells, mechanism of muscle cells, and also in bacterial motility as illustrated below, it has not been used to study cell reaction to topography.

It is important to study the viscosity of the medium when study the reaction of cells to topography because gap formed between lower side of the cells and the topography. This gap may contain a highly viscous medium, which affects the movement of molecules into and out of the cell. This may be an explanation for the hypothesis that cells react with planar topography more easier than they can react to rough topography. Alternatively it may increase the cell energy expenditure so much that it can not move.

The role of viscosity in protein dynamics

As has been discussed, the level of macromolecules, consisting of proteins, lipoproteins, and polysaccharides, determines the viscosity of the body fluids. Various studies have shown that solvent viscosity affects protein dynamics and reactions as reported by Tuvia et al in 1997. They have shown that the protein activity of the cell membrane is affected by the extracellular fluid macroviscosity (EFM), modified by macromolecular cosolvents as occurs in the body fluids.

There is also a possibility that the effect of medium viscosity on the enzymatic reaction involves the modulation of dynamic properties of membrane phospholipids. Yedgar et al (1987) suggested that medium viscosity is a regulator of very low density lipoprotein produced by cultured hepatocytes, where their secretion and synthesis are inversely proportional to the extracellular fluid viscosity. They considered the possibility that the mechanism of this extracellular effect on cell

function involves modulation of cell membrane components. They studied the effect of medium viscosity on the activity of phospholipase A2 (PLA2), an enzyme present in the cell surface membrane, and the activity has been correlated with cellular secretion. They found that culture medium viscosity inhibits the activity of PLA2 in the plasma membrane of cultured liver cells, concomitantly with the inhibition of lysosomal enzyme and lipoprotein secretion. It was also found that the degradation of liposomal phosphatidylcholine by soluble snake venom PLA2 is inversely proportional to the solvent viscosity, but there is no significant effect of the solvent viscosity on the phospholipid bilayer observed in their study.

Viscosity and plasma

Viscosity effects have been studied in blood plasma as well as in the extracellular fluid. Plasma viscosity is elevated in various pathological states, due to increased levels of protein and other macromolecules (Yedgar and Reisfeld, 1990). They found that secretion of lipoproteins and lysosomal enzymes by liver cells is inhibited as a function of the medium viscosity. Correspondingly, elevation of the plasma viscosity of hyperlipidemic rats reduced lipoprotein level. Elevated viscosity of blood and plasma is considered a risk factor for cardiovascular diseases and has been studied mainly in relation to microcirculation and hemodynamics (Tuvia et al, 1997).

Viscosity and blood cells

In addition there are many studies on the effect of medium or plasma viscosity on blood cells, such as Hovav et al's study in 1987. They studied the effect of medium viscosity on lysis of red blood cells (RBC's) by snake venom phospholipase A2 (PLA2). PLA2 and Ca^{++} were applied to cells suspended in viscous medium to induce hemolysis. It was found that hemolysis is inhibited in direct proportion to increasing viscosity of the extracellular fluid. This phenomenon was observed with aggregated as well as disaggregated RBC's. In the presence of Ca^{++}-cell-enzyme complex that was suspended in viscous medium, RBC lysis was also inhibited as the medium

viscosity was increased. The authors proposed that the viscosity of the cell surface aqueous environment regulate the action of PLA2 on RBC's membranes.
Kon et al in 1987, studied quantitatively the effect of shear force (depending on shear rate and the viscosity of the extracellular medium) and the hematocrit of erythrocyte suspension on RBC deformation using a cone-plate rheoscope with various kinds of cells. They found that the increase of suspension viscosity at higher hematocrits (Hts) generally enhanced the ellipsoidal deformation of cells, in the same manner as increasing the suspending medium viscosity of a diluted cell suspension.

Viscosity and muscle cells

The greatest effect of viscosity is to be expected during motion. Chase et al carried out the studies of the effect of medium viscosity on mechanisms of muscle cells in 1998. They stated that the highly dynamic muscle contraction might be influenced by viscosity of the medium surrounding the myofilaments. It was found that there was a linear dependence of 1/viscosity for both unloaded shortening velocity and also the kinetics of isometric tension redevelopment; these effects were unrelated to variation in solution osmolarity or inhibition of force. Thus they illustrated that the predominant effect of bulk solution viscosity is on an aspect of actomyosin function other than imposing a resistive force that oppose filament sliding. Their results implicate cross-bridge diffusion as a significant limiting factor in cross-bridge kinetics and demonstrate that viscosity is a useful probe of actomyosin dynamics.

Viscosity and bacterial motility

The effect of medium viscosity was also observed in lower organisms such as bacteria. Armitage and Packer in 1998, concluded in their study of bacterial motility that increasing the medium viscosity (by adding Ficoll to the medium) can also be used to measure the power output of motor. When the viscosity is increased too much it leads to cell is no longer able to translate, and there is no more cell body wobble. This is called the stall torque of the motor.

Viscosity and synaptic receptors

Kullmann et al, 1999 had shown that raising the viscosity of the extracellular medium could modulate the diffusion coefficient, providing an experimental tool to investigate the role of diffusion in activation the synaptic and extrasynaptic receptors in the brain.

Conclusion

In general, the extracellular fluid viscosity may play an important role in regulation of cellular and biochemical processes, Thus viscosity may also affect other fundamental properties of the cells.

Therefore, viscosity needs to be carefully considered in experimental design and interpretation of reaction of cells to topography.

AIMS OF THE PROJECT

The purpose of this research is to investigate the effect of the diffusion processes on the reactions of cells to topography, because diffusion is linked to the viscosity of the medium and to the shape of the extracellular space around the cell, which in turn could affect reaction of cells to topography.
It is well known that cells react to topography by various changes in their activity and morphology, these changes may be effects by the changes in medium viscosity. For example the diffusion out/in of a chemical might be easier on a grooved surface.

One suggestion has been that cells spread on topography may readily because diffusion under them is altered. Medium must be pushed out as cells spread over a surface help the cells. This could be expected because cells might fail to conform to a deep topography as well as they do to a planar surface. If diffusion is important viscosity should be important as well because diffusion rates and the viscosity of the medium are closely linked.
Therefore the reaction of cells to groove/ridge topography in media with different viscosity was examined in this project.
If diffusion processes are involved in the reactions high viscosity media should alter cell reaction to topography. The simplest feature of the reaction of cells I have examined is the morphological reaction of changes in cell length and width.

I have also looked at the gap (the separation distances between cells and substratum) using Interference Reflection Microscopy (IRM).
In addition, the hypothesis that cells do not conform to varied topographies as closely as they would to planar surfaces was tested, work with IRM shows this.
The effect of the viscosity of the medium on the cytoskeleton was also observed using Fluorescence microscopy.

Finally, using phase-contrast microscopy, cell movement on topography in the presence of medium of high viscosity was recorded and analyzed.

Chapter Two

Materials and Methods

CELLS AND CELL CULTURE MATERIALS

CELLS:

1. Epitenon cells:

Epitenon fibroblasts were isolated from rat flexor tendons of male Sprague Dawley rats according to the method described by Wojciak and Crossan, 1993. The cells were cultured in BHK 21 medium.

2. Mouse Endothelial B10D(2) cells:

There were isolated from mouse using the method reported in laboratory by Prof. Adam Curtis as described below:

Originally these were isolated from murine B10D2 strain pulmonary cells by growing explants of lung tissue in the Ham's F10 medium until appreciable outgrowths had developed. The explants were then removed manually. The outgrowth cells were grown to near confluency and then trypsinised to form cell suspensions.

Macrophages remained adherent at this stage and the cell suspension appeared to contain a mixture of fibroblasts, epithelia and endothelial cells. When these cells had grown to confluency they were trypsinised and the cell suspension treated with Dynal magnetic beads (450 grade) bearing the *Swainsonia* lectin specific for mouse endothelia. These beads were prepared in-house. The cells were incubated with a 100:1 ratio of beads to cells for 1 hour at 37^0 C. Then magnetic separation was started and the fibroblasts and epithelia, which had not attached beads, were removed. The beaded cells were placed in culture and as they divided they emerged from their bead coats and spread out as colonies of cells. These were grown to confluency, subcultured and the bead extraction repeated.

The cells were then subcultured again and the following tests used to establish their identity. They were found to be LDL-receptor positive, Swainsonia-lectin binding

positive, Factor VIII positive, to have Weibel-Palade bodies, to have normal chromosome counts and appearance, to be cytokeratin-negative and to have 'cobblestone' morphology at confluency though arcuate morphology at low density. Collagen types were consistent with an endothelial nature.
These tests were carried out by Professor A.Curtis and the line then maintained in the laboratory by repeated passage and/or storage at -70^0 C.

EPITENON CELL CULTURE

Epitenon cells (isolated from the surface of rat flexor tendons as mentioned before) were shown to be in cell cultures consisted mainly of flbroblasts-like type B cells (70%) and macrophage-like type A cells (Wojciak and Crossan, 1993). The respective cell types in the synovium and epitenon were morphologically indistinguishable (Wojciak and Crossan, 1993).

GROOVED SUBSTRATA

In recent years, the microfabrication technologies have been employed by biologists to examine cell behaviour (Burnette, 1986a and b; Dunn and Brown, 1986; Clark et al, 1987, 1990, 1991; Hirono et al, 1988; Wood, 1988). Therefore topographic cues have been shown by various studies to guide cell growth, migration, guidance and orientation (Clark et al. 1987; Stothard et al. 1995).
Although the chemistry of the material could be affecting the behaviour of the cells (Curtis and Clark 1990), it is necessary to examine the types and the sizes of structures that affect cell behaviour *in vitro* to determine a mechanism for guidance, and to show whether or not topographical environments found in nature could act as cues (Clark et al., 1990).

Multiple Grooved Substrata

The behaviour of cells on multiple parallel grooved substrata is now well developed and when compared with the behaviour of the same cell types on flat surfaces shows a marked difference in responses (Curtis and Wilkinson 1997).
Currently the material of choice for topographic control of cellular behaviour *in vitro* is fused silica. Different types from these materials of topography have been studied include v-shaped (Ohara and Buck, 1979; Dunn, 1982; Brunette, 1986; Brunette, 1988; Chehroudi et al, 1990) steps and holes, plane or groove and ridges (Wood, 1988; Clark et al; 1990; Meyle et al, 1991). But the best known structures which orientate cells and induce their directional movement are multiple grooved substrata (Curtis and Clark, 1990; Curtis and Varde, 1964).
Wojciak et al in 1995 enhanced the use of multiple grooved substrata to facilitate cell movement and to promote tendons healing *in vitro.*

Fused silica

Denbraber et al in 1995, modified silica grooved and plane surfaces by exposure to UV, which affected surface energies, but did not alter topographical reaction.
The choice of substrate is important. As cells typically have low optical contrast, phase-contrast microscopy is normal used. Glass does not etch well and so fused silica is a good choice for the substratum for experiments on the behaviour of cells on patterned surfaces consisting of grooves and ridges (Curtis and Wilkinson, 1998). Therefore it is preferred to make structures normally for *in vitro* experiments using fused silica as a substrate; usually there will be a thin covering of protein in the surface because the cell is unlikely to be in direct contact with the surface of the silica (Wilkinson et al, 1998).

The work in this project deals with the effect of topography on cell behaviour on plane and multiple parallel grooved substrata. All grooved structures were fabricated from fused silica, silicon oxide (SiO2).

MEDIA:

1. ECT Media:

Eagles Water * (dH_2O)	134 ml
BHK 21 Media (10 concentrate)	16 ml
Tryptose Phosphate	20 ml
Calf Serum(Gibco)	20 ml
Sodium bicarbonate**(sol.7.5%)	7 ml
Antibiotic mix***	5 ml

This medium is CO_2 buffered after adding cells.

2. Ham's F10 Media:

22mM Hepes	180ml
Ham's F10 (*10concentrate)	20ml
Foetal calf serum	6ml
Sodium bicarbonate**(sol.7.5%)	2.5m
Antibiotic mix***	5ml
ITS	2ml

***Eagles Water:**

High quality water (double distilled, RO quality) aliquoted into 134 ml in 250 ml bottles and autoclaved. Store at 4^0C.

****Bicarbonate:**

7.5% solution of Sodium hydrogen carbonate in high quality water.

For 1 litre

Sodium bicarbonate	75 g

When fully dissolved sterilise by Millipore ultra-filtration and dispense in 20ml aliquotes. Store at room temperature.

***Antibiotics:**

A mixture of Glutamine, Penicillin, Streptomycin and Fungizone (Amphotericin B) all from Gibco/Life technology, UK.

For 1 litre

Glutamine	29.2 g

Sterile, filter and aliquot into Plastic Bottles at 150 ml per bottle.

For 1 volume

Glutamine	150 ml
Fungizone	12.5 ml
Penicillin/Sterptomycin	100 ml

Mix and aliquot sterile in a laminar flow hood.

Hepes Saline:

A calcium and magnesium free HEPES buffered balanced salt solution, suitable for washing cell monolayers prior to trypsinisation.

For 1 litre

Sodium chloride	8 g
Potassium chloride	0.4 g
d-Glucose	1
Hepes	2.38 g
0.5% Phenol red	2 ml

Adjust to pH 7.5 with 5M NaOH . Dispense in 100ml aliquots and autoclave, store at 4^0c after sterilisation.

(Hepes and Phenol red were from Sigma, UK; all other materials were from BDH-Merck Ltd., Lutterworth, Leics., England).

Hepes Buffered Water:

A non-isotonic buffer solution for dilution of cell culture media x10 concentrates.

For 1 litre

Hepes	5.25 g

Adjust to pH 7.5 with 5M NaOH. Dispense in 180ml aliquots and autoclave. Store at 4^0C after sterilisation.

Tryptose Phosphate:

29.5 g powder in 1 litre water, dispensed in 20ml aliquots, autoclaved, and store at 4^0C.

Buffer: Phosphate Buffered Saline (PBS):

For 1 litre:

NaCl	9.86 g
KCl	0.25 g
Na_2HPO_4 (anhydrous)	1.44 g
KH_2PO_4	0.25g

pH adjusted to 7.2.

BSA / PBS

1g of Bovine serum albumin (1% BSA, Sigma) in 100ml PBS.

PBS / Tween 20

0.5% of Tween 20 (Polyoxyethylene-sorbitan monolaurate, Sigma, USA) in 100ml PBS.

Buffered Formalin (Formal Saline 4%w/w):

For 1 litre:

100 mls of formaldehyde (solution 38% w/w) (BDH-Merck Ltd., Lutterworth, Leics., England) were added to 900 ml of phosphate buffered saline [BDH-Merck] and the pH adjusted to 7.4.

4 % Formaldehyde + Sucrose

4 ml formaldehyde in 100 ml PBS + 2 % sucrose (Fisher Scientific, UK).

Permeabilisation buffer

Sucrose	10.3 g
NaCl	0.292 g
MgCl2	0.06 g
Hepes	0.476 g

In 100 ml distilled water.

pH adjusted to 7.2.

then 0.5ml TritonX was added.

TISSUE CULTURE

Tissue Culture Vessels:

-Tissue culture polystyrene petri-dishes (60mm) were obtained from Corning, New York, USA.

-Tissue culture polystyrene flasks 25cm^2 from Iwaki, Japan.

-Glass bottles from100-1000cm^2.

Substratum Patterning:

2.1.Grooved Slides:

Microgrooved structures of fused silica (quartz), silicon oxide (SiO2), slides with grating width a bout 5μm-10μm made by Electronics and Electrical Engineering Department in Glasgow University.

All quartz materials obtained from Whitehart Scientific Lens co., Newcastle.

2.2.Planar (non-grooved) Structures:

Normal glass cover-slips 22x22mm obtained from (BDH-Merck eurolab).

2.3.Grooved Cover-slips:

Multi grating pattern on 22x22mm cover-slips of fused silica (quartz) (obtained from Whitehart Scientific Lens co., Newcastle) with grating width of about 5μm-10μm, and depth from 280-420nm for used with IRM and TIRFM.

All these structures were cleaned by soaking in Caro's acid (3:1sulphuric acid 98%: hydrogen peroxide 30%) for 10min followed by a rinse in R.O. (reverse osmosis) water then sterilized in 70% ethanol.

2.4.Planar Quartz Cover-slips:

Fused silica 22x22mm cover-slips plane used for TIRFM experiments, obtained from Whitehart Scientific Lens co., Newcastle UK.

Slides:

-Normal slides and cavity slides obtained from different companies.
-Fused silica slides used for TIRFM, obtained from Whitehart Scientific Lens co., Newcastle, UK.

Solutions for detaching cells from culture surfaces:

1.Versene:

A Hepes buffered, balanced salt solution containing 0.5mM EDTA (Ethylenediaminetetra acetic acid disodium salt)[Sigma,UK] for use in conjunction with Trypsin for the dissociation of cell monolayers

For 1 litre

Sodium chloride	8 g
Potassium chloride	0.4 g
d-Glucose	1 g

Hepes	2.38 g
EDTA	0.2 g
0.5% Phenol red	2 ml

Adjust to pH 7.5 with 5M NaOH . Dispense in 20ml aliquots and autoclave, store at 4^0c after sterilisation.

2.Trypsin:

Sterile 2.5% w/v trypsin (Gibco/life Technologies 1:250) in normal PBS saline.
Adjust to pH 7.5, dispensed in 2 ml aliquots, and stored at -20^0C.
Activity: 1g of Trypsin will digest 250g of casein substrate under conditions of USP for protease activity of pancreatin ($+25^0$ c for 10 min at pH 7.6).
0.5 ml of this solution was added to 20 ml Versene.

Stains

1. Coomassie blue

1g Coomassie Brilliant blue R-250 [Sigma, UK] dissolved in 1L of 500ml methanol / 500ml water and 70ml acetic acid) used to stain cells for the first section of experiment.

2. Phalloidin

Stock solution: FITC- Phalloidin (Sigma) 1mg + 1ml methanol kept at -20^0C.

Reagents used to change medium viscosity:

1. High molecular weight polymers used experimentally:

Viscosity of the medium was varied by adding to the normal media (ECT or Hams'F10) various macromolecules that differ in size, chemical nature, and in their capacity to increase fluid viscosity these were:

-Carboxymethylcellulose [BDH laboratory supplies, UK] has high viscosity, with concentration of 0.5g in 100ml of ECT media.

-Methyl Cellulose with concentration of:

0.5g in 100ml ECT or Ham's F10 media

1g in 100ml ECT or Ham's F10 media

2g in 100ml ECT or Ham's F10 media

3g in 100ml ECT or Ham's F10 media

4g in 100ml ECT or Ham's F10 media

-Ficoll with molecular weight 400,000 [Sigma, UK] with concentration of:

0.5g in 100ml ECT or Ham's F10 media

2g in 100ml ECT or Ham's F10 media

4g in 100ml ECT or Ham's F10 media

6g in 100ml ECT or Ham's F10 media

8g in 100ml ECT or Ham's F10 media

-Dextran with molecular weight 503,000 [Sigma, UK] with concentration of:

1g in 100ml ECT or Ham's F10 media.

2g in 100ml ECT or Ham's F10 media

4g in 100ml ECT or Ham's F10 media

-Dextran with molecular weight 2,000,000 [Sigma, UK] with concentration of:

0.5g in 100ml ECT or Ham's F10 media.

2g in 100ml ECT or Ham's F10 media.

-Polyvinylpyrrolidone [Sigma, UK] with molecular weight 40,000 with concentration of 0.5g in 100 ml ECT or Ham's F10 media.

-Polyvinylpyrrolidone [Sigma, UK] with molecular weight 360,000 with concentration of 0.5g in 100 ml ECT or Ham's F10 media.

2. Lower molecular weight polymers used as a control:

-Dextran [Sigma, UK] with molecular weight 9,000 with concentration of:

0.5g in 100 ml ECT or Ham's F10 media

2g in 100 ml ECT or Ham's F10 media

4g in 100 ml ECT or Ham's F10 media

-Ficoll [Sigma, UK] with molecular weight 70,000 with concentration of:

0.5g in 100 ml ECT or Ham's F10 media

2g in 100 ml ECT or Ham's F10 media

4g in 100 ml ECT or Ham's F10 media

Reagents were used for TIRF microscopy:

Texas red labeled dextran (Tetramethylrhodamine isothiocyanate-Dextran) TRITC [Sigma, UK] with molecular weight of 65,000 and concentration of 20mg in each 1ml PBS.

INSTRUMENTS

1. Measurement Instruments

1.1. Viscometer:

The U-tube Ostwald viscometer was been used to measure the medium viscosity. The viscometer is shown in figure 3.

1.2. Refractometer:

An Abbé Refractometer (figure 4) was used to measure the refractive index for all media used in IRM experiments.

The Abbé Refractometer is a precision optical instrument comprising two glass prisms, between which the test liquid is sandwiched as a thin film, illuminated by white light source (a lamp).

1.3. Osmometer:

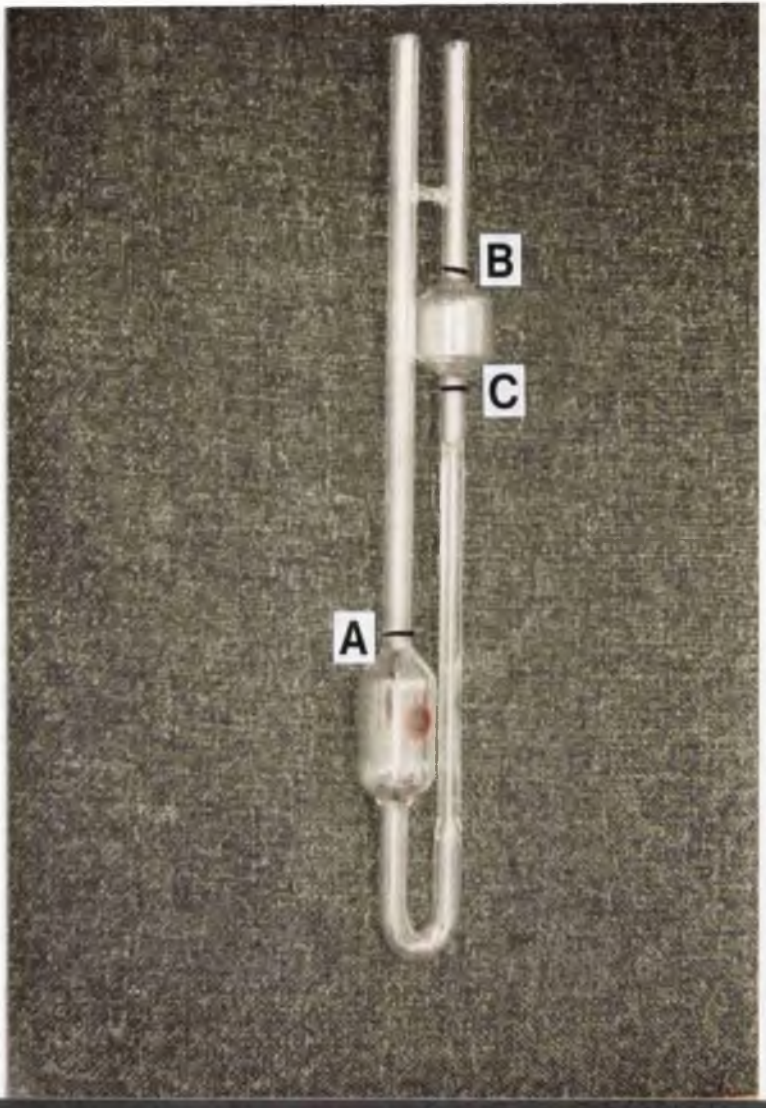
The GONOTEC digital micro-osmometer (under the supervision of Biochemistry lab in Yorkhill Hospital, Glasgow) was used to measure the media osmolarity at freezing point depression. I thank the laboratory of Biochemistry Department in Royal Hospital for Sick Children, where the osmolarity measurements were carried out.

Figure 3:

The U-tube Ostwald viscometer that was used to measure the media viscosity. It was filled the liquid at the level A in the wide arm (when standing vertical under temperature control). The liquid was drawn up to a level above B, released, and the time of flow between the marks B and C was measured.

Figure 4:

An Abbé Refractometer that was used to measure the refractive index.


B
C
A

2. Microscopy:

2.1. Light Microscopy:

2.1.1. Equipment used to measure cells by light microscope:

Light microscope Leitz Wetzlar Model Diavert, Germany.

Micrometer Eyepiece.

Stage Micrometer.

All Micrometers supplemented by Graticules Ltd, Tonbridge Kent, UK.

2.1.2. Equipment used for specimen imaging:

Light microscope Leitz Wetzlar Model Diavert, Germany.

Computer imaging system composed of:

-CCD Camera Model C545-01 (Hamamatsu Photronics, UK).

-Image processor Model C5510 (Hamamatsu, UK).

-Grystalscan computer (Gateway 2000).

-Monitor Sony Model PVM-142M2E (Hamamatsu, UK).

2.1.3. Equipment used for Video recording:

-CCD video camera CV-M50 connects to Zeiss microscope.

-JVC Television + Panasonic Time-lapse video.

-COHU high performance CCD camera connects to Diavert microscope, Germany.

-JVC Television and Panasonic JVC video.

-Excel computers program to analysis the measurements data of the cells moving.

2.2. Internal Reflection Microscopy (IRM)

Ordinary IRM microscope built in the laboratory from a Vickers M 17 microscope with a neutral beam splitter replacing a dichronic filter and collimation system, see Figure 8 in next chapter.

2.3. Fluorescence Microscopy

A Vickers M 17 fluorescence microscope with a Hamamatsu CCD camera.

2.4. Temperature controlled IRM:

A modified IRM (figure 5) was built from normal IRM surrounded by a box to keep the experiment temperature near 37^0 C. The medium of high viscosity was aspirated during live imaging using a modified petri-dish which was connected to a syringe by a thin tube (figure 6).

2.5. Total Internal Reflection Microscope (TRIFM):

Built by me TIRF microscope used as described below and shown in figure 7.

2.5.1. Suggested optical set up of the TIRF microscope:

Figure 7 schematically shows the experimental set up. A Helium-Neon laser (Coherent, UK), random polarised light at 543.5 nm with a constant power output of 0.3 mW was used. This laser has been assembled in robust larger diameter (44.5mm) aluminium cylindrical housing. It operates with TEM_{00} low-divergence circular (Gaussian) collimated beams and less than 1 % RMS noise. The laser beam travels through an a variable- angle laser mount (laser diameter 44.5mm and angular adjustment $\mp 17^0$).

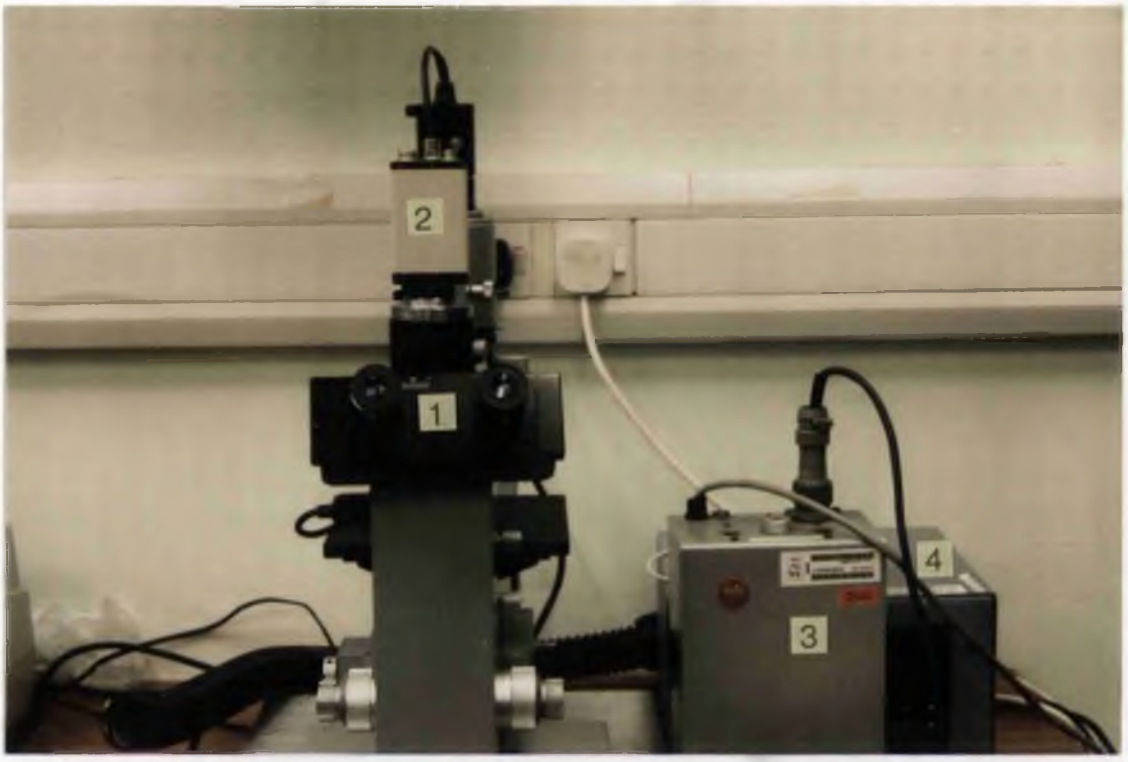
After passing a glass large prism mounted in between a set of mounted iris diaphragm, 25 mm in diameter, minimum aperture 0.8 mm (Coherent), which is used to direct the laser light and increase the central intensity into an inverting quartz prism (I C Optical systems LTD UK) at an angle above the critical angle of total internal reflection. The fluorescence signal, collected by a x50 oil immersion objective (Vickers, England) passes a tube lens which have a visible band-pass yellow filter (CWL=589.6nm, effective index=2.10 n*, 25.4mm in diameter, min. peak trans.50%, with 3 cavities, Ealing, UK) is used to separate fluorescence from scattered incidence light and is finally focused onto a high resolution CCD (Model C545-01 Hamamatsu photronics, UK). For data analysis an image processing system has used (Model C5510 C, UK), which runs Hamamatsu camera program on a GrystalScan computer (Gateway 2000). The whole system has been mounted on laser top optical base plate (660 x 900mm with a thickness of 50mm, Coherent UK).

Figure 5:

A temperature controlled IRM that was used for live IRM images, 1 IRM microscope, 2 CCD camera, 3 Power supply, 4 Temperature controller the whole system were surrounded by box to maintain temperature near 37^0 C.

Figure 6:

Equipment used with temperature controlled live IRM to change media. A plastic syringe, B modified petri dish contain a central hall adhesive to glass cover slip, C plastic tube.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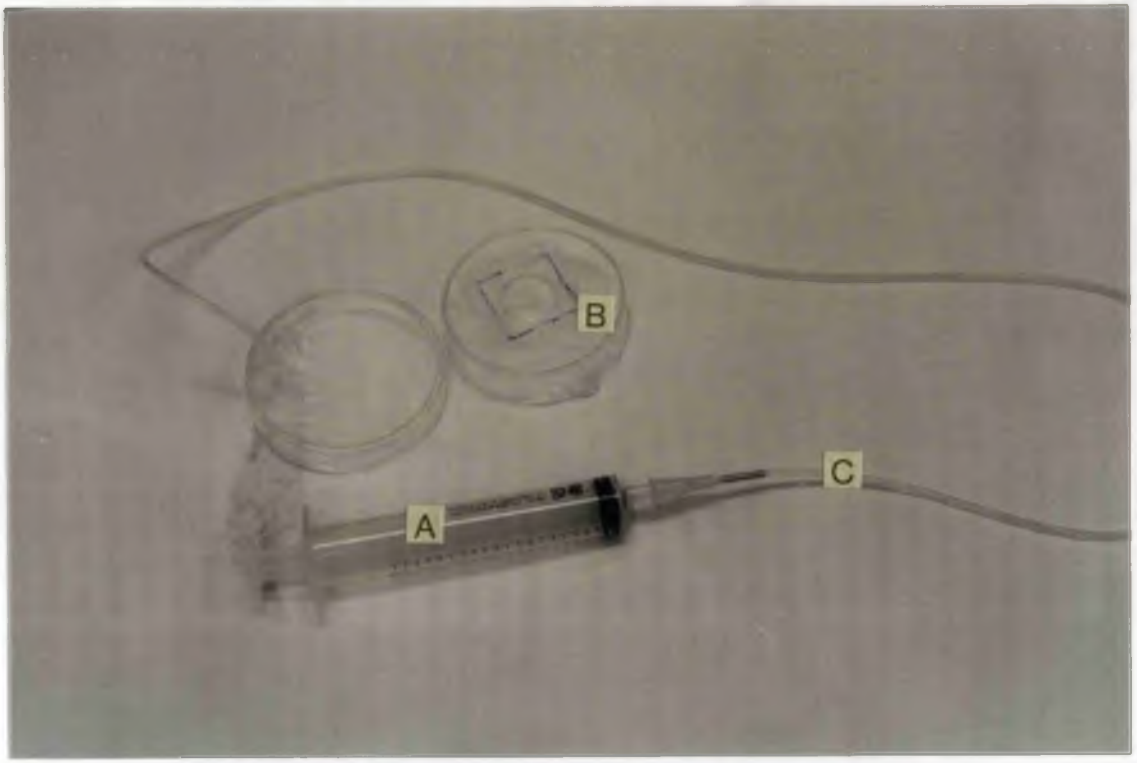
1
4
3


B
C
A

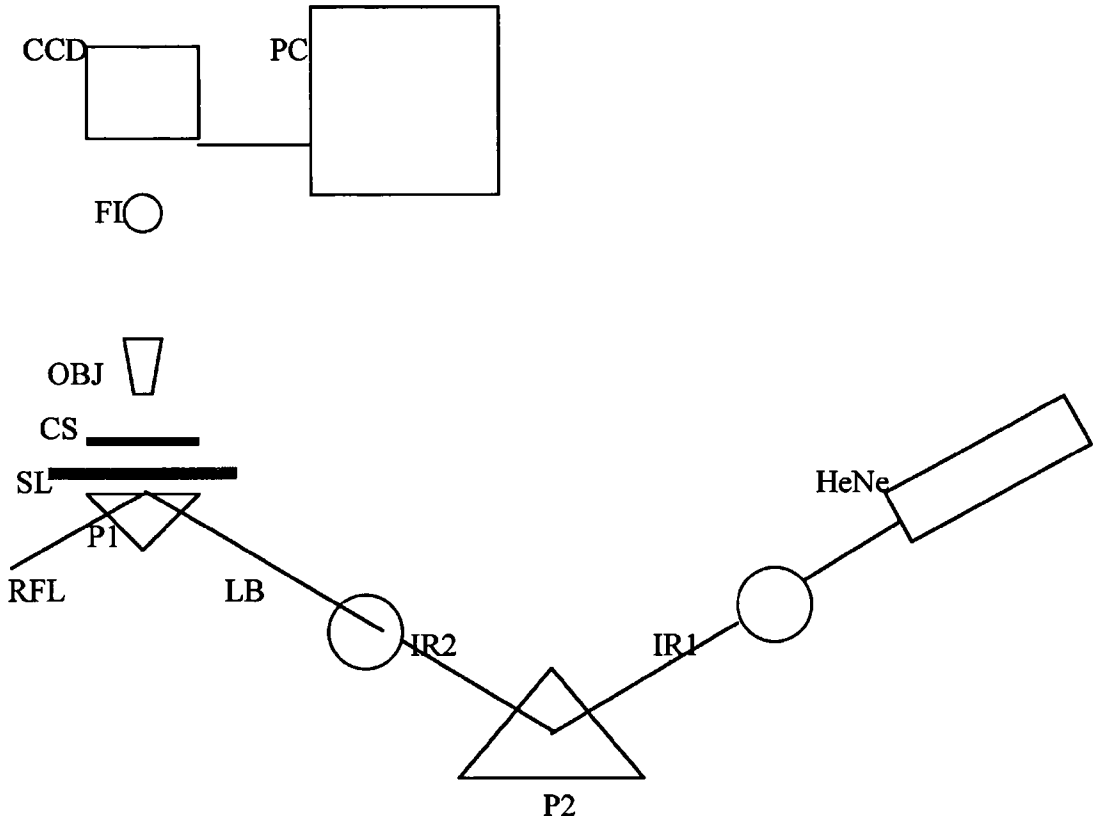


Figure 7:

Experimental setup of the TIRF microscope.

HeNe: Helium-Neon laser supply, CCD: Hamamatsu CCD camera, FL: yellow filter, OBJ: objective, CS inverted coverslip with specimen, SL slide on oil immersion, P1: quartz prism, RFL: reflected light beam, LB: laser beam, IR1 & IR2: set of iris diaphragm, P2: intermediate glass prism.

Preparation of cells for microscopic examination

General preparation of cells:

Mouse epitenon or mouse endothelial B10 D2 were obtained as mentioned page on 34. Cells were grown in polystyrene flasks 25cm^2 and passage biweekly. Cells were suspended in phosphate-buffered saline (PBS), centrifuged, resuspended in culture medium, and plated onto plain and grooved substrata as required for each experiments.

Light microscopy examination and imaging:

Each group of cells were plated onto grooved substrata with groove width 5μm-10μm in high viscosity media (medium viscosity was altered by adding high molecular weight molecules to the normal media as mentioned before) and control media and incubated at 37^{0}C for 24h. Cells were fixed in 4% formaldehyde for 5 min at room temperature after double washing with 1x PBS each for 5 min. Then cells were stained with Coomassie blue for 5-10 min followed by repeated washing with tap water.

The cell length and greater width of 50 cells each in experimental and as control were measured in each kind of media using an ocular micrometer and stage micrometer. Results have been calculated and compared with the corresponding values from the statistical tables.

Images typically were recorded using a CCD camera and saved in PC computer. Images processed using NIH Image 1.62n3 computer program.

The data was analysed using stateView 4.02, CA-Cricket GraphIII and Excel computer programs.

Live phase-contrast microscopy examination and imaging:

Cells were cultured in normal media (ECT for epitenon cells and Ham's F10 for endothelial cells, B10D2) as a control on 5 μm grooved substrata in normal or modified petri dishes (Figure 6, page 51) under temperature control at 37^0C. Cells were examined using a 20x or 10x objectives by phase-contrast microscopy and video recorded for 24h, then the media replaced with high viscosity media and imaged for more 24h. To analyse the data 300-700 frame time-lapses were processed using NIH Image 1.62n3 and Excel computer programs.

IRM microscopy examination and imaging:

Cells were cultured in experimental high viscosity and control media (ECT for epitenon cells and Ham's F10 for endothelial cells, B10D2) on grooved substrata (5μm-10μm in width and 420-280nm depth) and planar quartz cover-slips for 24h or for only 3h. Then they were examined by Interference-reflection microscope IRM (Objective 50x) using cavity slides contains PBS. Cavity slides were used to avoid secondary reflection which appear if normal slides used with cover-slips to make IRM examination due to the proximity of the two glass surfaces with plane slides. Images were saved using CCD camera and PC. Data analysed and processed using NIH Image 1.62n3 computer program.

Live IRM microscopy examination and imaging:

Cells were allowed to grow in normal media (ECT for epitenon cells and Ham's F10 for endothelial cells, B10D2) for 24h in modified petri dishes. Then the cell were observed and video recorded for 30 min as a control then the media were changed to the experimental high viscosity media using a long tube connected with the media dish without touching the system. The changes were recorded by time-lapse video for three hours or more for different experiments. Sequences were exported as TIFF files, and further processed using Adobe Photoshop 3.0, NIH Image 1.62n3 and QuikTime movies.

All movies were recorded in CD Roms.

F-actin in cultured cells stained with fluorescently labelled Phalloidin

Cells were grown on grooved and flat surfaces in control and experimental media for 24h. Following this, they were rinsed with PBS (37^0C) then fixed with 4% Formalin in PBS + 2% Sucrose (37^0C) for 5 minutes. Cells were then rinsed 3 times with PBS. Cell permeabilisation was carried out using permeabilising buffer (4^0C) for 5 minutes. Then rinsed with PBS / BSA (37^0C) for 5 minutes. The cells were then stained with FITC-Phalloidin (Sigma Chemicals) at a dilution of 1:100 in PBS for 1 hour. Then rinsed 3 times each 5 minutes with PBS/ Tween 20. The specimens were then mounted on slides in Vectashield mounting medium for fluorescence (from Vector Laboratories, Burlingame, CA), with a cover slip. The F-actin was visualised using a Hamamatsu CCD camera on a Vickers M15 fluorescent microscope.

TIRF microscopy examination and imaging:

Test experiment:

Cells cultured on glass cover slips for 24h.
Specimen were fixed in 1% Glutaraldehyde for 10 min
Washed by BPS.
Stained with fluorochrome (TRITC-Dextran) for an hour or more.
Washed with BPS.

Regular experiment:

Cells (epitenon or endothelial) cultured in normal media (ECT for epitenon cells and Ham's F10 for endothelial cells, B2D10) on grooved or plane cover slips for 24 h. Fluorescent dye added to the specimen and observed by TIRFM.

Chapter Three

Special Methods

SPECIAL METHODS

INTERFERENCE REFLECTION MICROSCOPY (IRM)

History a critique:

Close contact of a cell with its substratum can be measured by means of interference-reflection microscopy (IRM). This technique, first used for this purpose by (Curtis 1964), examined the interference of the light reflected from the cell surface and from a substratum nearby.
After Curtis, IRM was used by Izzard & Lochner (Lochner & Izzard, 1973; Izzard & Lochner, 1976) to study the adhesion of cells to glass, then in 1979 Gingell and Todd provided a theoretical analysis in details.

The importance of IRM lies in its suitability for studying cell-substrate adhesion patterns in living, moving cells.

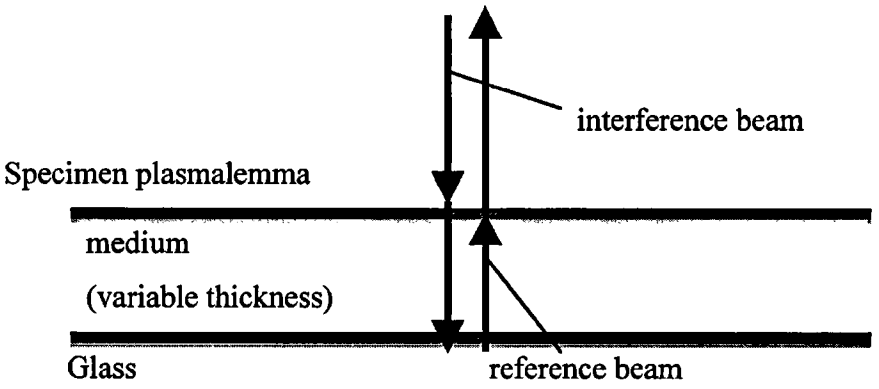


Figure 8:

The figure shows the interference technique of the IRM.

It is an interference technique where regions of the membrane in closest apposition to the substrate surface appear as reflectivity minima only under some conditions (Curtis, 1964, Gingell et al, 1985 and Burmeister 1998).

IRM images

Gingell and Todd in 1979 postulated that the interference reflection images are composed of reflected light modulated in intensity by interference of light from other reflecting interfaces that were nearby.

When using the (IRM) dark areas indicate a cell-substratum separation of less than 20nm and the grey areas indicate a separation of 20-80nm.

Gingell and Vince (1979) stated that IRM is probably incapable of distinguishing molecular contacts from gaps of less than 5nm. In fact according to Verschuren in 1985, as the limiting case of immediate contact is approached, there is a gradual decrease in sensitivity to wavelength and aperture, but there is no abrupt fall at zero separation distance. Since the optical path length within the medium layer between cell and glass is a function of the angle of refraction in the medium, the broad range of the angles of incidence within the illuminating cone produced at high illuminating numerical aperture (INA) results in a continuous series of overlapping interference patterns, which can cancel out each other almost entirely. Only for the zero-order fringe (optical bath difference, smaller than one wavelength) do these overlapping patterns converge; there is an increase in intensity for a gap increasing from 0 to ~ 100nm. Thus at high INA (>1) the higher-order interference fringes are lost, and the image is constituted solely from zero-order interferences, giving information on cell substrate separation within the range (Verschuren in 1985). Curtis 1964, saw 10nm contact between cell and substratum. These were latter termed focal contacts.

Qualitative and quantitative of IRM

IRM has been applied qualitatively to a large number of cell types, and it can view the two universal types of adhesion, focal contacts and close contacts.

Izzard & Lochner (1976), observed the distinct dark streaks of close contacts between cells and the substratum with the IRM and called them focal contacts (Truskey, 1992).

Truskey (1992) suggested, by using of IRM, that focal contacts are narrow (e.g.0.2μm x 10μm) and the closest regions of an adherent cell membrane to the substratum surface (within 10-20 nm). They are firm attachment structures that hold the cell in place and in its spread shape.

Close contacts are broad areas with membrane/substratum separations of 30-50 nm. They are weaker, but highly dynamic adhesions that sustain rapid movements of cells or cell parts over the substrate. Extracellular matrix contacts refer to regions of the cell membrane separated from the substratum by 100 nm or more (Burridge et al., 1988; Izzard and Lochner, 1976; Chen and Singer, 1982). IRM work on cell adhesion has been devoted to the cell-substratum interactions of fibroblasts *in vitro* and show hows that the separation between the under surface of a fibroblasts and the substratum is variable (Curtis, 1964; Izzard & Lochner, 1976; Garrod et al., 1981). Abercrombie (1980), suggested that focal contacts seem to contain concentrations of α-actinin, the molecule that is anchorage for actin in muscle cells.

Additional uses

IRM has been used to examine the association of specific proteins with focal contacts and to characterise cell migration (Burmeister et al, 1998).

Bereiter-Hahn et al (1979), proposed that IRM could be used quantitatively to determine refractive indices at points where the optical path difference is known. Where the cells are at least 50nm distant from the glass substrate, intensities are also influenced by that distance as well as by the angle of incidence of the light and the wavelength. The reflected intensity at the glass/medium interface is used as a standard in calculating the refractive index of the cortical cytoplasm. Refractive indices were found to be higher (1.38-1.40) at points of focal contact, where stress fibres terminate, than in areas of close contact (1.354-1.368). In areas of the cortical cytoplasm between focal contacts, not adherent to the glass substrate, refractive

indices between 1.353 and 1.368 were found. This was thought to result from a microfilamentous network within the cortical cytoplasm.
Many authors misused IRM method by using high INA. This problem was exhorted by the use of central beam stop as in the instrument commercial, may be light, where only opaque illumination was used.
A methodological improvement in the quantitative study of adhesion patterns and particularly the extent of the focal contact formation in different cell types and different situations, might come from the use of automatic image analysis in IRM (Bereiter-Hahn, 1977). In addition to the description of adhesion patterns in different cell types and the elucidation of the molecular events involved in the formation of specialized adhesive structures, IRM has also been used to study the role of long-rang forces, electrostatic repulsion and London-van der Waals' attraction, in cell-substrate interaction by a number of authors (Curtis, 1964; King, Heaysman & Perston, 1979; Gingell & Todd, 1977; 1980; Gingell & Vince, 1979, 1982; Vince & Gingell, 1982).

Finally, IRM can also be used to enhance contrast in images of fixed, stained or unstained preparations, such as histological sections, blood smears and chromosomes (Verschuren, 1985; Pera, 1979a,b).

These applications suggest that IRM is likely to be further developed in coming years, and will become of interest to an increasing number of microscopists (Verschueren, 1985).

Applications of IRM in cell biology

Many different cell types have now been studied with IRM, although fibroblastic cells have been studied in the most detail. Embryonic chick heart fibroblasts were used in the pioneering work of Curtis (1964) and Izzard & Lochner (1973, 1976), who defined the basic features of IRM images of living cells. IRM of chick heart fibroblasts has been used further to study the relationship between adhesion patterns and locomotion (Dunn & Heath, 1976; Dunn, 1979; Izzard & Lochner, 1980; Chen, 1981), contact inhibition of movement (Abercrombie & Dunn, 1975), the association

of adhesions with the microfilament system (Heath &Dunn, 1978; Heath, 1983) and the interrelationship of adhesion, spreading, growth and movement (Badley, 1980; Couchman & Rees, 1979a,b; Rees et al., 1979; Badley, et al., 1980).
Rees et al., 1977 discussed IRM images of rat dermal fibroblasts and baby hamster kidney (BHK) cells during detachment from glass. The role of fibronectin in locomotion and the anchorage of fibroblasts has also been studied using IRM (Couchman et al., 1982; Rees et al., 1982; Chandrasekhar et al., 1983; Sanders, 1984).

COMPARING INTERFERENCE REFLECTION MICROSCOPY AND TOTAL INTERNAL REFLECTION FLUORESCENT MICROSCOPY

IRM and TIRFM (Total internal reflection fluorescent microscopy) exhibit different spatial sensitivity as a function of separation distance. For IRM, the relative intensity is not sensitive to changes in the separation distances for values between 0 and 8 nm. Only above 8nm is the IRM sensitive to changes in separation distance. IRM cannot be used to accurately calculate membrane/substrate separation distance less than 8nm, an important region for analysis of bond interactions (Burmeister 1998). TIRFM is more sensitive to small separation distances with two other methods, surface plasmon resonance and fluorescence resonance energy transfer (FRET), are still more precise. IRM has the distinct advantage over TIRFM and FRET that it does not require the use of fluorescent markers to visualize cell contact regions, which eliminates the possibility of cellular damage caused by the presence of the fluorophores. However, IRM images are produced using only 1% of the illuminating incident light. The absence of fluorescent labels has the disadvantage that images do not contain any information from specifically labelled molecules in the contact regions (Burmeister 1998).
One advantage of TIRFM is that only a very thin layer at the bottom of the cell is illuminated; thus an excellent fluorescent signal-to-noise ratio is obtained and photo damage is minimised. In addition, the light emitted by the fluorophore will

exponentially increase as it approaches the coverslip and can facilitate detection of fusion (Toomre et al, 2000).

Burmeister (1998) found by using TIRFM that cell adhesion to polymers depends upon a number of variables amenable to experimental manipulations including: (a) the surface properties; (b) the density and affinity of adhesion molecules on the surface; (c) covalent and non-covalent interactions between the cell and surface molecules; (d) the time of interaction between the cell and the surface; and (e) signalling events within the cell to promote or inhibit cell spreading.

Recently, TIRFM has been used for very interesting applications in the observation of processes near the cell membrane (Rohrbach, 2000).

PHASE-CONTRAST MICROSCOPY

Phase-contrast, developed by Zernike in the 1930s (Zernike, 1955), has become standard equipment in all biological laboratories, because of its simplicity and its performance in observing details of living cells (Verschuren, 1985).
When light passes through a living cell, the phase of the light wave is changed according to the refractive index of the cells and the thickness.
As the phase of the light is altered by its passage through the cell, small phase differences can be made visible by exploiting interference effects using a phase-contrast microscope and the differential-interference-contrast microscope (Alberts et al, 1989).

Images enhanced and analyzed by electronic techniques

In recent years digital imaging systems and the associated technology of image processing have had a major impact in light microscopy. The limitations of microscopes due to imperfections in the optical system can be overcome by attaching highly light sensitive video cameras to a microscope. It is then possible to observe cells for long periods at very low light levels, thereby avoiding the damaging effects of prolonged bright light and heat.

Numerical aperture (NA) limits the optical resolution of a microscope (Abbe limit). In other words, the contrast is low for a low-resolution image. This problem can beovercome by using electronic techniques, for example using small pixels to give high resolution, since the resolution in the digitised image depends on the number of pixels (Wilkinson and Schut, 1998) and especially grey level expansion to raise contrast.

Because images produced by video cameras are in electronic form, they can be readily digitised, fed to a computer, and processed in various ways to extract latent information. By using video systems linked to image processors, contrast can be greatly enhanced so that the visual limitations in detecting small differences in light intensity are overcome (Alberts et al, 1989).

CCD cameras can be divided roughly into two groups: video cameras and scientific cameras. Video cameras deliver a continuous analogue output signal that conforms to a specified standard. The video signals can be displayed using a video monitor or digitised by any compatible frame grabber. Scientific cameras deliver a single or multiple high quality images on command. They do not obey any standard and are available with a wide variety of options. These options include pixel sizes, image dimension, readout rate, integration time, cooling, quality grade and dynamic range (Alberts et al, 1989).

Although this processing also enhances the effects of random background irregularities in the optical system, this 'noise' can be removed by electronically subtracting an image of a blank area of the field. Small transparent objects then become visible that were previously impossible to distinguish from the background (Alberts et al, 1989).

Chapter Four

Results

RESULTS

1. THE EFFECT OF MEDIUM VISCOSITY AND OSMOLARITY ON CELL REACTIONS TO TOPOGRAPHY

1.1. VISCOSITY MEASUREMENTS

This chapter records viscosity and osmolarity measurements:

Viscosity measurements of the media used

Measurements show in Table 3 indicates that the viscosity of the medium significantly increased as the concentration of high molecular weight molecules increased in the solution compared with the viscosity of water, ECT media and the low molecular weight molecules that were used as controls.
Therefore, the possibility that the effect of medium viscosity on the reaction of cells is associated with the osmotic pressure must be considered. To examine this hypothesis the medium osmolarity was measured for all used media.

The real viscosities of the media were obtained (centi Poises) from Fick's law.

Equation 1:

$$\eta / P = At$$

Therefore (η) is the viscosity, P is the density, A is a constant related to the viscometer tube dimensions and t is the flow rate of solution in the tube.

Table 3: Viscosity measurements:

Media name	Viscosity [cP]	Time of flow solution between B-C by sec.				
Water as a control	0.69	12	11	11	12	11
ECT normal media	0.75	13	12	13	12	12
0.5%Dextran 9,000	0.75	12	13	12	12	12
Low 2%Dextran 9,000	0.82	13	12	13	13	13
Low 4%Dextran 9,000	0.88	14	14	14	14	14
Low 0.5%Ficoll 70,000	0.75	12	12	12	12	12
Low 2%Ficoll 70,000	0.82	13	13	13	13	13
Low 4%Ficoll 70,000	0.94	15	15	14	14	15
4%Dextran 503,000	2.58	42	40	41	41	40
2%Dextran 503,000	1.57	25	25	25	25	25
1%Dextran 503,000	1.01	15	16	16	15	15
0.5%Dextran 2000,000	1.07	17	17	17	17	16
2%Dextran 2000,000	2.33	37	37	36	38	37
0.5%Polyvinylpyrrlidone 360k	1.26	20	20	20	21	20
0.5%Polyvinylpyrrolidone 40k	0.82	13	13	14	13	13
0.5 % Carboxymethylcellulose	48.09	760	825	728	776	736
3%Methylcellulose(MC)	<1131.55	More than 4 hours				
2 %Methylcellulose(MC)	9.37	148	150	150	149	149
1%Methylcellulose (MC)	3.08	49	49	49	49	50
0.5 %Methylcellulose	1.57	25	24	24	25	25
0.5% Ficoll 400,000	0.82	13	13	12	12	13
2%Ficoll 400,000	1.07	16	18	16	18	17
4%Ficoll 400,000	1.32	20	21	20	22	21
6%Ficoll 400,000	2.26	36	36	36	36	36
8%Ficoll 400,000	2.58	41	41	40	41	41
Olive oil	31.43	500	505	490	498	500
Dimethylpolysiloxane (silicone oil) 5 centistoke	3.52	57	56	55	55	56
Dimethylpolysiloxane (silicone oil) 100 cetistoke	76.88	20m.23s			20m.23s	

1.2. OSMOLARITY MEASUREMENTS

The osmolarity measurements:

The osmolarity measurements were measured using the Gonotec digital micro-Osmometer (030) to measure freezing points depression, the results are shows in Table 4:

Table 4: The osmolarity measurements

Media name	Concentration	Osmolarity (m Osm/kg)
Control ECT media		330
Ficoll 70,000	4%	338
Ficoll400,000	1%	334
Ficoll400,000	4%	356
Ficoll400,000	8%	358
Dextran 503,000	1%	330
Dextran 503,000	4%	330
Dextran 2000,000	0.5%	329
Dextran 2000,000	2%	335

This indicates that there are no significant differences of the osmotic pressure from medium to medium between control ECT media and other experimental high viscous media.

This suggests that any effects found will not be due to osmotic effects.

Here I would like to mention with grateful thanks Mr John Black from laboratory of Biochemistry Department in Royal Hospital for Sick Children, who did these osmolarity measurements.

2. Recovery experiments

Of interest is the finding that cells are still alive after incubated in high viscosity medium as obtained from the recovery experiments as show in Figure 9 & 10 Cells after recovery grow and appear to restore their validity. This confirms the suggestion that cells strong enough to live in a viscous conditions. Bares 2.5 µm.

Figure 9:

Images show Epitenon cells

1- Control ECT medium for 24h

2- 2%Dextran 2000,000 for 24h

3- ECT as a recover for 24h

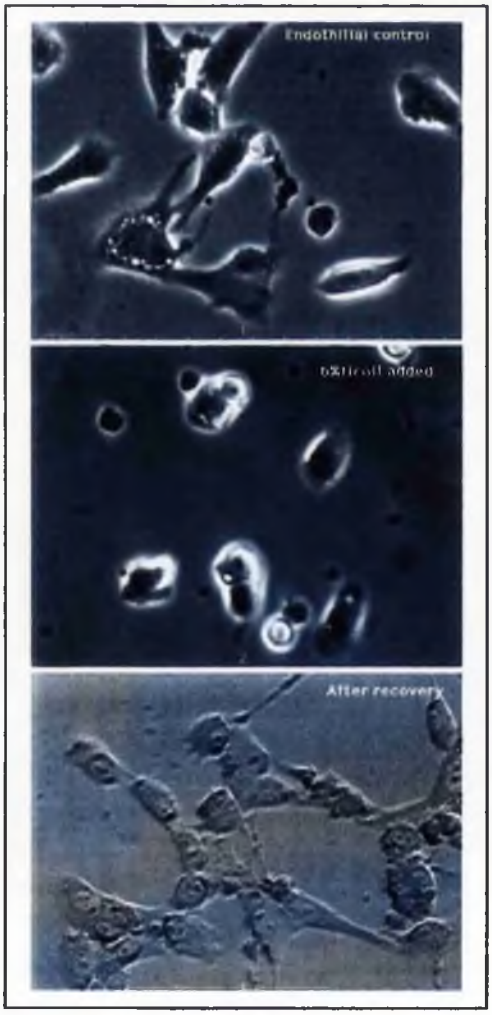


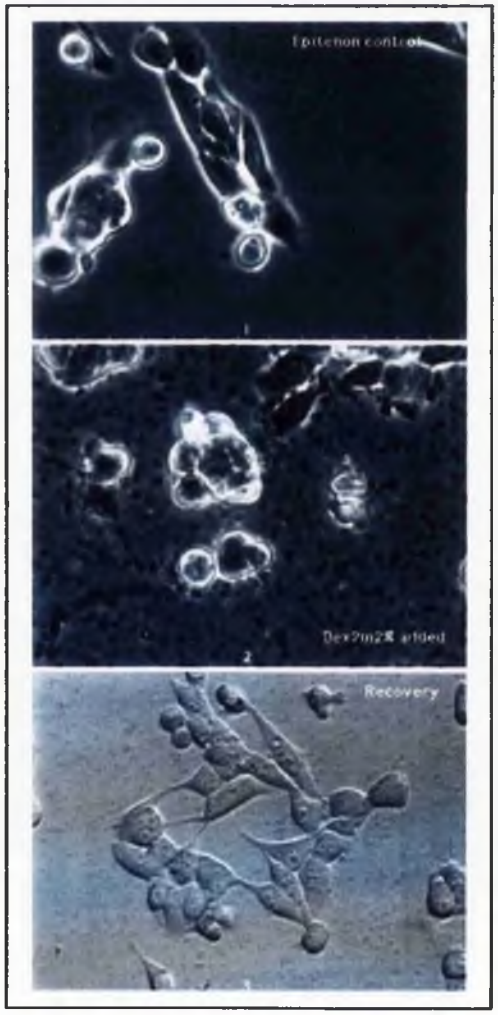
Figure 10:

Images show Endothelial cells B10D2

1- Control Ham's F10 for 24h

2- 6% Ficoll 400,000 for 24 h

3- Ham's F10 as a recover for 24h.



3. MORPHOLOCICAL REACTIONS

Morphometry:

The reaction of cells to topography is reduced so the average of length of cells decreased as medium viscosity is increased for all reagents as shown in the related Figures 11-30 Tables (5-12).

Experiments were done on 5-10μm groove ridges and on planar controls. The media used are of various viscosities.

3. 1. A study of the morphology of Epitenon cells on grooved topography:

3. 1. 1. The effect of high molecular weight molecules:

3. 1.1.1. By studying the effect of different concentrations of Dextran (with high molecular weight) on the length of the epitenon cells, it was found that the low concentration of Dextran did not affect significantly the viscosity of the media, and it did not affect the epitenon cell morphology significantly.

But the higher concentration of Dextran ($P<0.0001$ in all), the higher is the viscosity of the media and there is significant effect on the length of cells, but not on the width as shown in Table 5, Figure 11, 18 and 19.

3. 1.1.2. By studying the effect of different concentration of Ficoll (400,000) on the length of the epitenon cells. It was found that the length of the cells was affected by Ficoll concentration as shown in Table 6, Figure 12, 21 and 22.

The following equation was used to calculate the colloid osmotic pressure of Ficoll, alternatively from the real osmolarity measurements which was mentioned before:

Equation 2: $\Pi = M_s R T$ II (Osmotic pressure)

$T = 310$ T (temperature)

$$M_s = \frac{\text{gm/litre}}{\text{mol.wt}} = \frac{80}{400{,}000} = 0.0002$$

$R = 0.08206$ R (gas constant)

$\Pi = 310 \times 0.0002 \times 0.08206 = 5 \times 10^{-3}$ ats

Comparing with 0.145M Nacl in vertebrates

$\Pi = 0.145 \times 310 \times 0.08206 = 3.6$ ats

This indicates that there is no significant effect of the osmotic pressure on the size of the cells in these experiments.

3. 1.1.3. By studying the effect of different concentration of Methylcellulose (MC) on the length of epitenon cells, it was found that the length was affected by the concentration of the MC as shown in Table 7 Figure 13 and 23.
This result indicates that the length of the epitenon cells decreases with increasing of concentration.

3. 1.1.4. The other high molecular weight molecules, which were used in this experiment (Carboxymethylcellulose and the two different concentration of polyvinylpyrrolidone), affected the length of epitenon cells in the same way as shown in the Figures 20 and 24.

3. 1. 2. The effect on the width of the epitenon cells:

While the width of the epitenon cells showed no effects when the medium viscosity increased using low or medium concentration of high molecular weight reagents, the width of the cells showed a similar significant increase when using high concentration of the high viscous reagents, cells in this medium appeared rounded, non-spreading with approximately same width and length.
In the very high concentration of reagents as seen with MC (3%) the cells appear shrunken with small width (see Fig 23).

3. 1. 3. The effect of low molecular weight molecules used as a control:

3. 1.3.1. By studying the effect of low molecular weight polymer (e.g. Dextran with mol.wt 9,000) on the length of epitenon cells I found that there is no significant effect on the length of epitenon cells incubated in low Dextran at different concentrations as shown in Table 8 and Figure 14 and 25.

By comparing this experiment with the previous experiments on high molecular weight dextran we show that the epitenon cell length is affected by the medium viscosity and not by the chemistry of the polymers because all the five polymers of high molecular weight had the same effects at similar viscosities.

3. 1.3.2. By studying the effect of low polymer (Ficoll with mol. Wt. 70,000) on the epitenon cells length, the results are similar to low molecular weight dextran, see Table 9 Figure 15 and 26.

Student's *t* test was applied to compare the cell length of control and experimental cells. The test indicates that the effect of high viscosity medium on cell length was statistically significant ($P<0.0001$).

These results indicate that when there is no significant increase in the viscosity of the medium there is no effect on cell length. But when the viscosity was increased (viscosity $\geq$ 1c.P) appreciably the cell length was reduced ($P <0.0001$).

To confirm my results more I used another type of cells, murine capillary endothelial B10D2 and the same results that were observed with epitenon cells were shown. See Table 10-11 and Figures 16 -17 and 27-29.

For all subsequent results (Table 5- 12) P values are obtained form StatView 4.02 Program and *t* values calculated according to Snedecor and Cochran, 1980 as:

$$t = b / sb$$

Where, *b* is the regression coefficient of the viscosity and *sb* is the Standard deviation of the regression coefficient obtained by StatView 4.02 program.

Table 5: The effect of high molecular weight Dextran on epitenon cell length :

Dextran conc.	Average length (μm) of 50 cells	Standard deviation	P-value exp. / ctrl.
Control 1	81	42	
Dextran 1% M.wt503,000	71	27	0.8391
Dextran 2% M.wt503,000	70	27	0.8424
Control 2 Dex2000,000	96	33	
Dextran 4% M.wt503,000	44	41	<0.0001
Dextran0.5% Mw2000,000	51	26	<0.0001
Dextran 2% Mw2000,000	50	27	<0.0001

Figure 11:

The curve shows that the epitenon cell length decreases if the Dextran concentration increases. Standard deviation of the regression coefficient *(sb)* = - 0.781 and $t = 25.18$

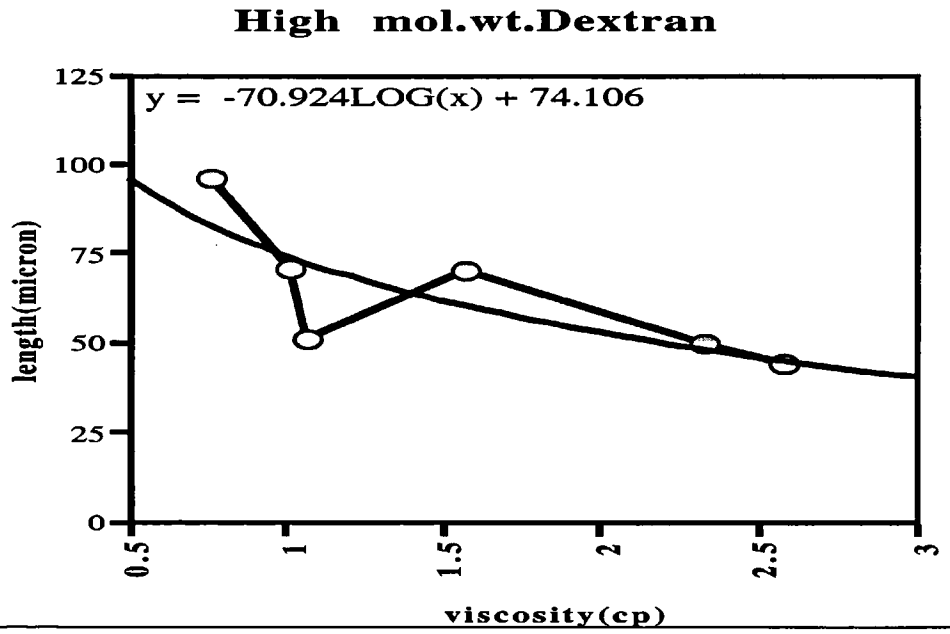


Table 6: The effect of Ficoll (mol.wt.400,000) on epitenon cell length:

Cone.	Average length (μm) of 50 cells	Standard deviation.	P-value cont. &exp.
Control 1	94.42	43.2	
Ficoll4%	35.13	15.4	<0.0001
Ficoll6%	24.93	6.6	<0.0001
Ficoll8%	21.81	8.4	<0.0001
Control2	103	43	
Ficoll0.5%	80	22	0.0008
Ficoll2%	59	21	<0.0001

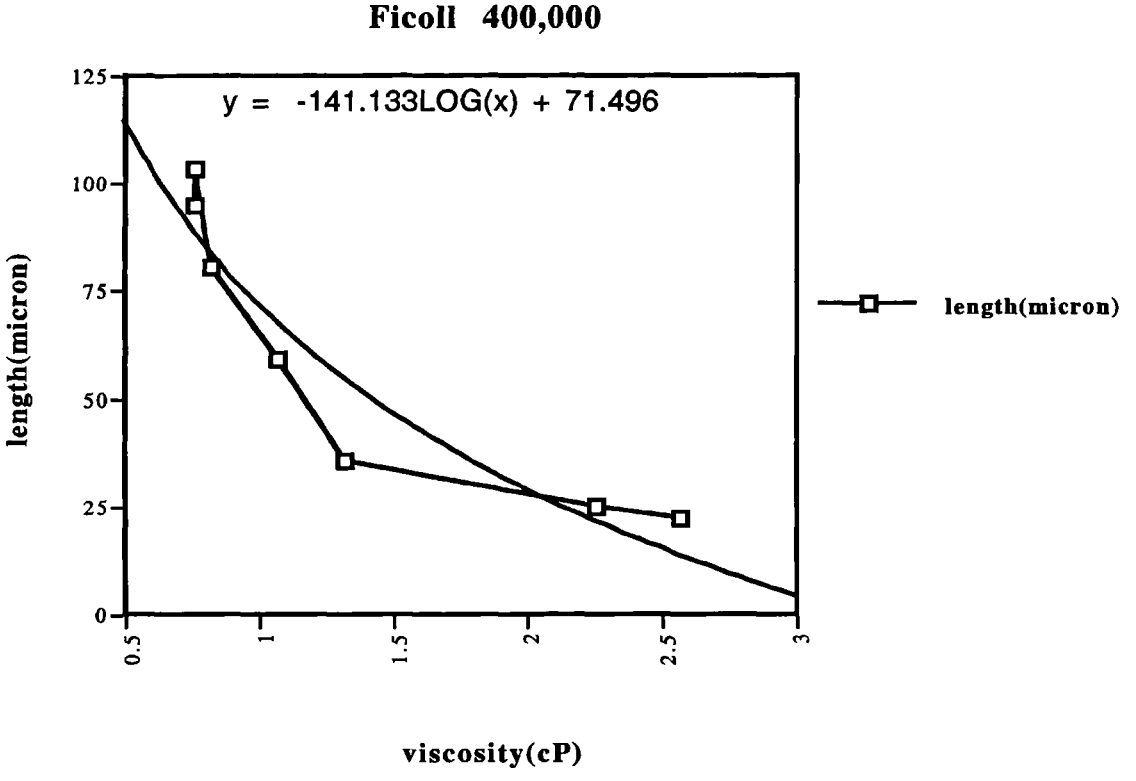


Figure 12:

The curve shows the relation between the cell length and the viscosity of ficoll medium. Standard deviation of the regression coefficient *(sb)* = - 0.888 and $t = 53.05$

Table 7: The effect of Methyl cellulose on epitenon cell length:

MC conc.	Average length (μm) of 50 cells	Standard deviation	P-value
Control 1	119.9	49.98	
MC 0.5%	69.4	30.4	<0.0001
MC 2%	68.9	25.7	<0.0001
Control 2	81.35	41.99	
MC 1%	40.32	29.2	<0.0001
MC 3%	10.4	8.05	<0.0001

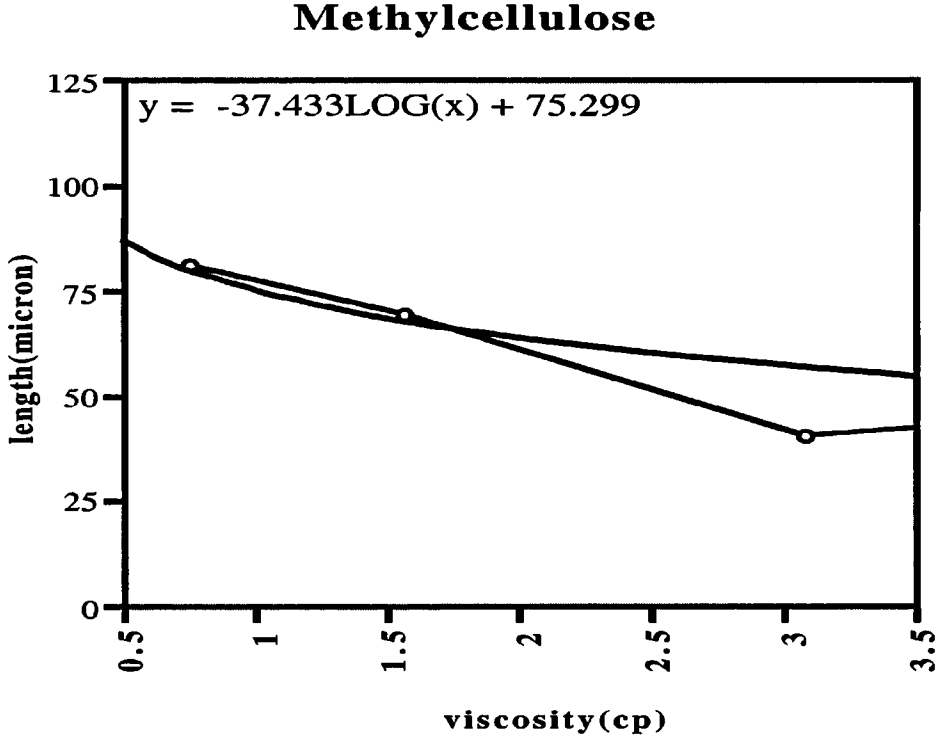


Figure 13:

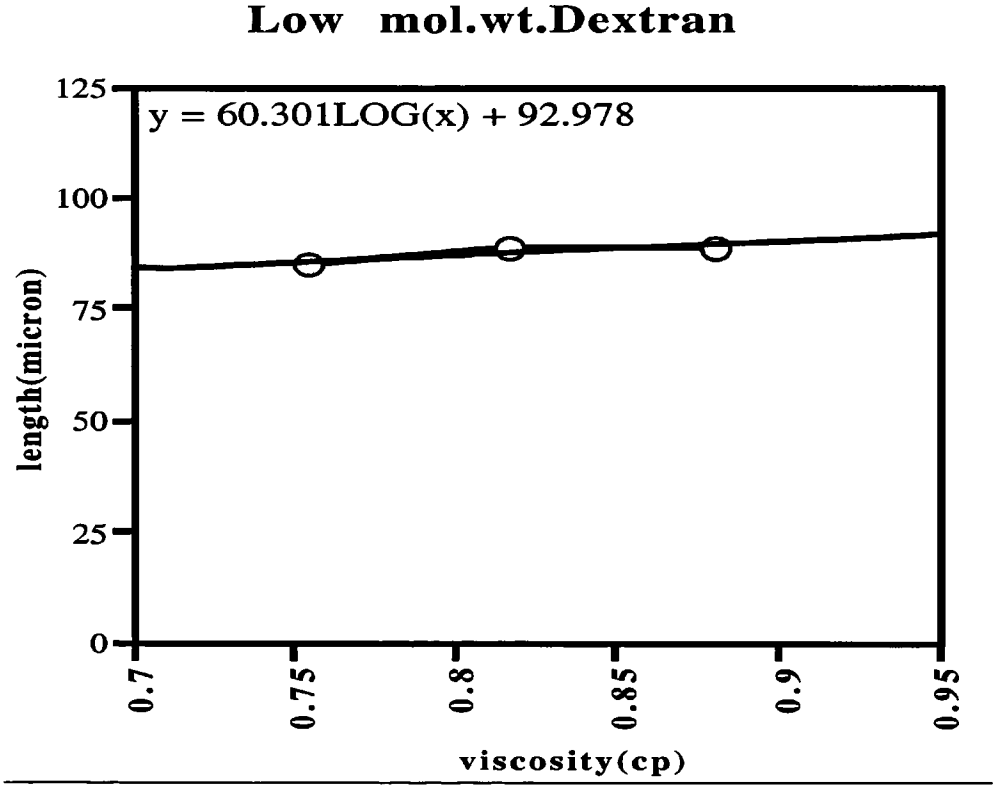
The curve shows the relation between MC concentration (viscosity) and the cells length. Standard deviation of the regression coefficient *(sb)* = - 0.794 and $t = 3.569$

Table 8: Low mol. Wt. (9,000) Dextran used as a control:

Dextran conc.	Average length (μm) of 50 cells	Standard deviation	P-value
Control1 normal ECT	93	39	
Dextran0.5% Mol.wt 9,000	85	29	0.012
Dextran 2% Mol.wt 9,000	88.9	38	0.39
Dextran 4% Mol.wt 9,000	89	26	0.004

Figure 14:

The curve shows the relationship between viscosity (different concentration of low mol. wt. Dextran) and epitenon cell length. Standard deviation of the regression coefficient *(sb)* = - 2.687 and $t = 0.054$



3. 2. A study of the morphology of Endothelial cells grown in highly viscous media on grooved topography

3. 2. 1. The effect of Ficoll (mol.wt.400,000) on endothelial cell length:

By studying the effect of different concentration of Ficoll on the length of the endothelial cells. It was found that the length of the cells was affected by Ficoll concentration as follows:

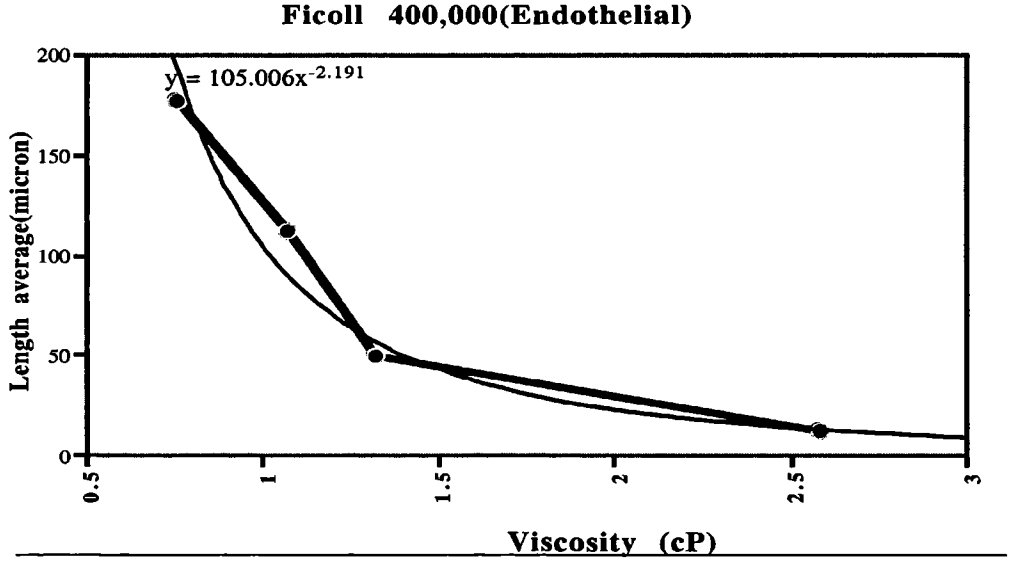
Table 10: Ficoll effect on endothelial cells:

Ficoll Conc.	Standard deviation.	Average length (μm) of 50 cells	P-value
Ham's F10	72.5	178.2	
Ficoll 2%	45.7	113.5	0.002
Ficoll 4%	53.6	50	0.037
Ficoll 8%	4.7	13	<0.0001

This indicates that the length of the endothelial cells decrease with the increasing of Ficoll concentration as show in the Figure.

Figure16:

The curve shows the decrease of endothelial length average with increase of medium viscosity. Standard deviation of the regression coefficient *(sb)* = - 0.873 and $t = 90.72$



3. 2. 2. The effect of Dextran (mol.wt.2000.000 and 503,0000) on endothelial cell length:

By studying the effect of different concentration of Dextran on the length of the endothelial cells. It was found that the length of the cells was affected by Dextran concentration as follows:

Table 11: Dextran effect on endothelial cells:

Dextran Conc.&Mw.	Standard deviation.	Average length (μm) of 50 cells	P-value
Control Ham's F10	81.4	177.9	
Dextran0.5% Mw.2000,000	73.6	142.9	0.485
Dextran 1% Mw.2000,000	50.6	94.5	0.001
Control Ham's F10	57	163.7	
Dextran2% Mw.503,000	62.3	127.7	.564
Dextran 4% Mw.503,000	43	87	.055

This indicates that the length of the endothelial cells decreases with increasing Dextran concentration as show in the curve on page 77:

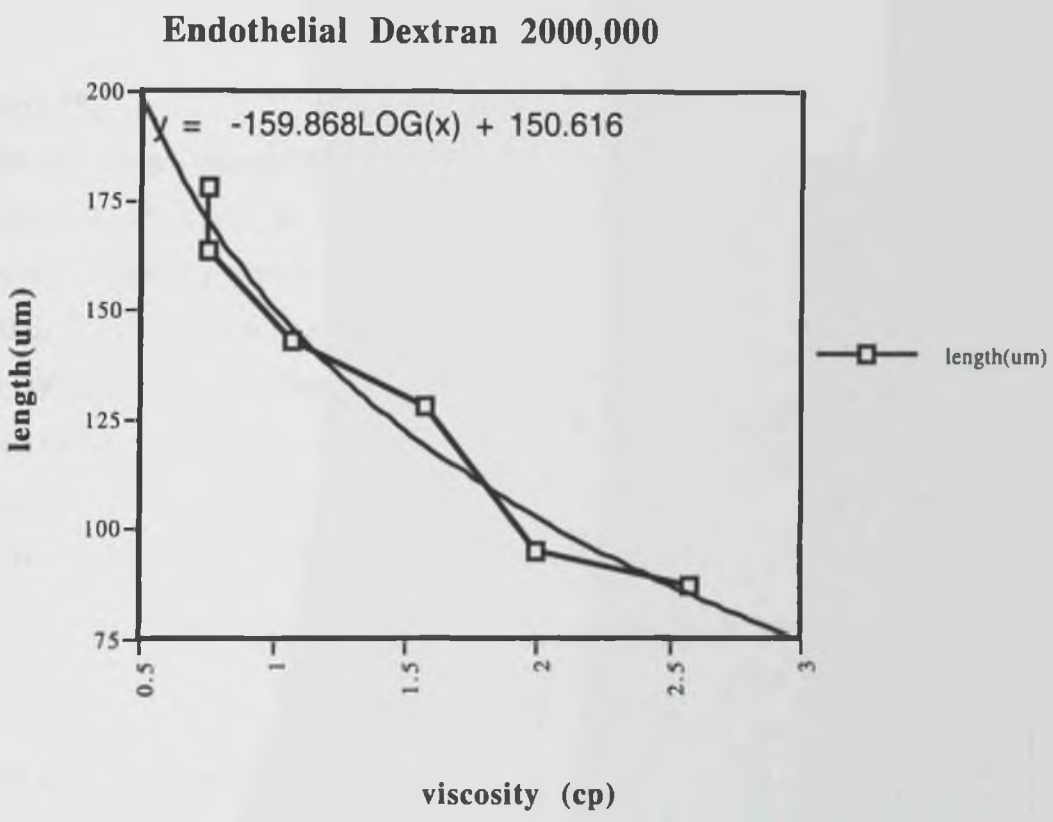


Figure17:

The curve shows the decrease of endothelial length average with the increase of medium viscosity. Standard deviation of the regression coefficient *(sb)* = -0.795, and $t = 53.05$ is calculated from Snedecor and Cochran, 1980 as example:

$$t = \frac{b}{sb} = \frac{-42.171}{-0.795} = 53.05$$

Therefore, *b* is the regression coefficient of the viscosity *sb* is the Standard deviation of the regression coefficient as StatView 4.02 program.

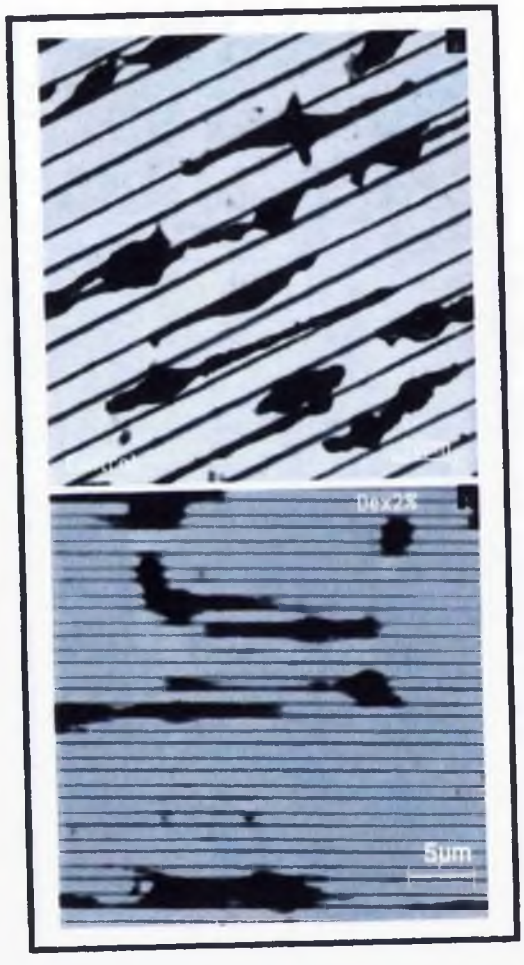
All graphs have been fitted by least squares method using computer program.

Figure 18:

Light microscopy images (20x-Objective) of Epitenon cells grown on quartz grooved topography with depth 5-8μm and 5-10μm width in Dextran with mol.wt 503,000 and Control ECT media for 24h. Fixed and stained with Coomassie blue

a -Control

b -Experimental Dextran with conc.2%.



Scale bars = 5 μm

Number above scale bar indicates groove width.

Figure 19:

Light microscopy images (20x-Objective) of Epitenon cells grown on quartz grooved topography with depth 5-8μm and 5μm width in Dextran with mol.wt 2000,000 and Control ECT medium for 24h. Fixed and stained with Coomassie blue

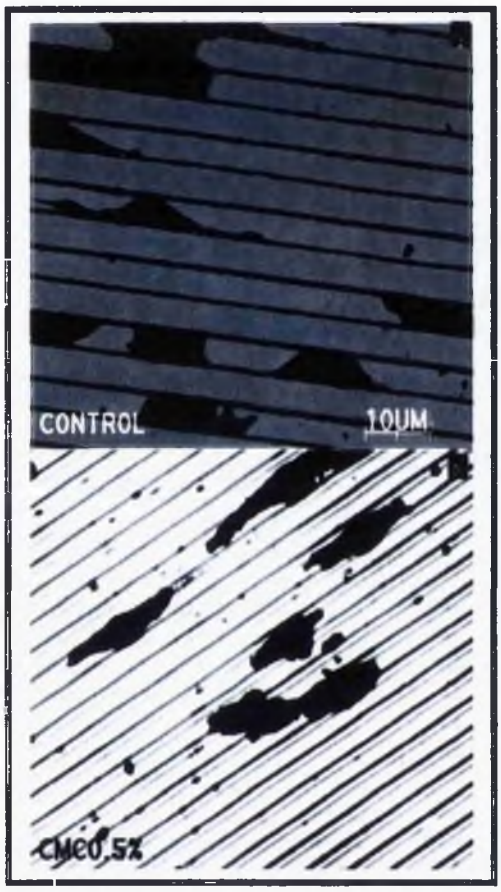
1. Control.
2. Dextran conc.0.5%
3. Dextran conc.2%

Scale bars 5μm for all images.

Number above scale bar indicates groove width.

Figure 20:

Light microscopy images (20x-Objective) of Epitenon cells grown on quartz grooved topography with depth 5-8μm and 10μm width in Carboxymethylcellulose and Control ECTmedium for 24h.
Fixed and stained with Coomassie blue



a- Control

b- Carboxymethylcellulose conc.0.5%

Scale bars 5μm for all images
Number above scale bar indicates groove width.

Figure 21:

Light microscopy images (20x-Objective) of Epitenon cells grown on quartz grooved topography with depth 5-8μm and 5-10μm width in Ficoll 400,000 and Control ECT medium for 24h.
Fixed and stained with Coomassie blue

1. Control.
2. Ficoll conc.0.5%.
3. Ficoll conc.2%.
4. Ficoll conc.4%.

Scale bars = 5 μm
Number above scale bar indicates groove width.

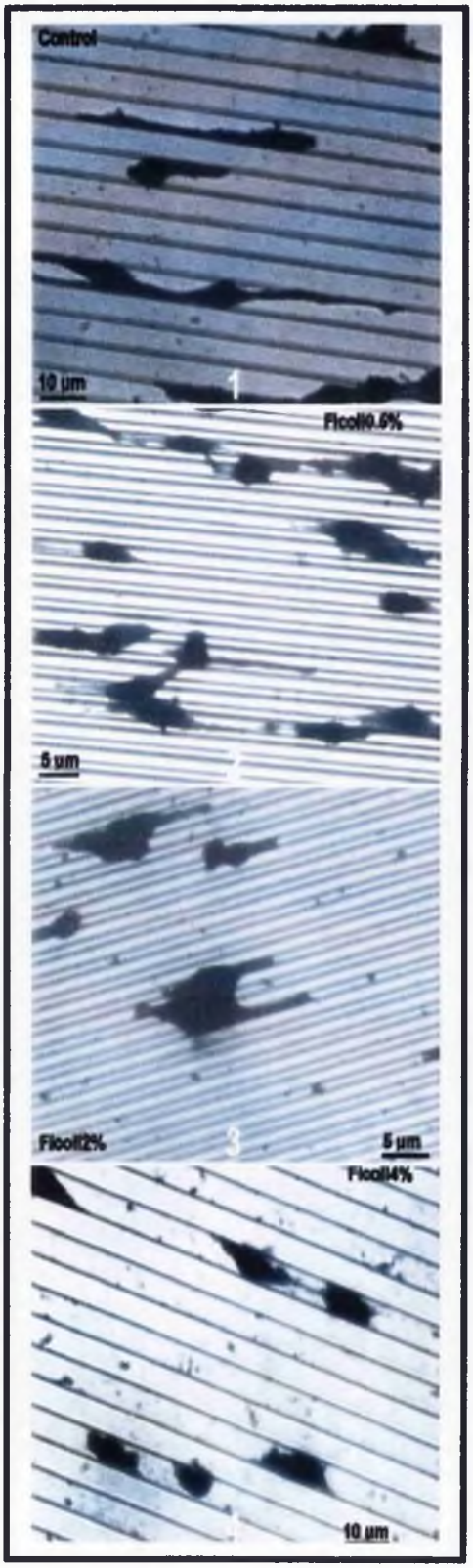


Figure 22:

Light microscopy images (20x-Objective) of Epitenon cells grown on quartz grooved topography with depth 5-8μm and 5-10μm width in Ficoll 400,000 and Control ECT medium for 24h.
Fixed and stained with Coomassie blue

a- Control
b- Ficoll conc.6%
c- Ficoll conc.8%

Scale bars = 5 μm
Number above scale bar indicates groove width.

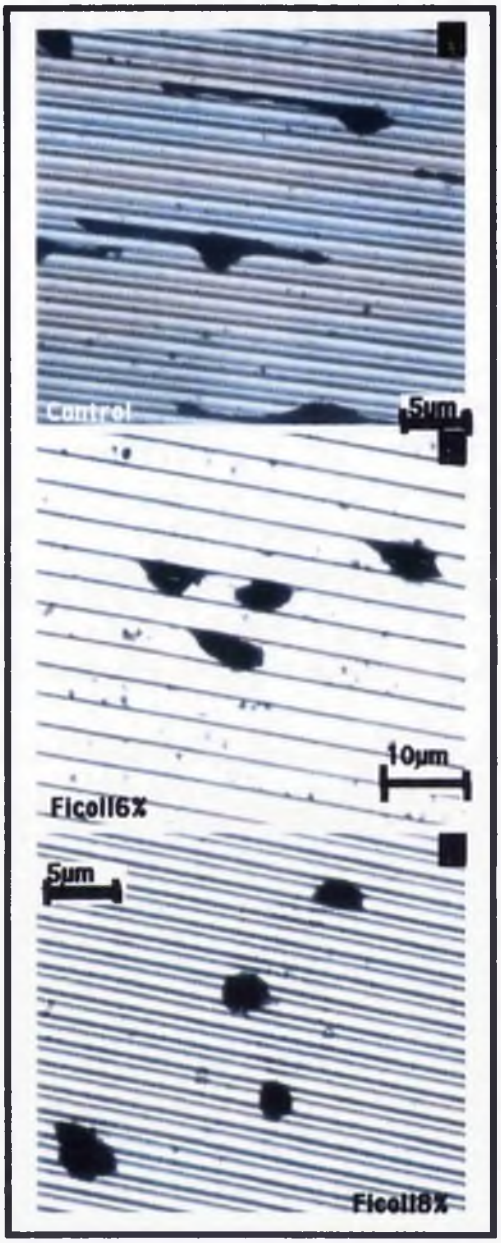


Figure 23:

Light microscopy images (20x-Objective) of Epitenon cells grown in Methylcellulose for 24h on quartz grooved topography with depth 5μm and 5-10μm width.

Fixed and stained with Coomassie blue

a. MC conc.1%

b. MC conc 3%

Scale bars for all images 5 μm

Number above scale bar indicates groove width.

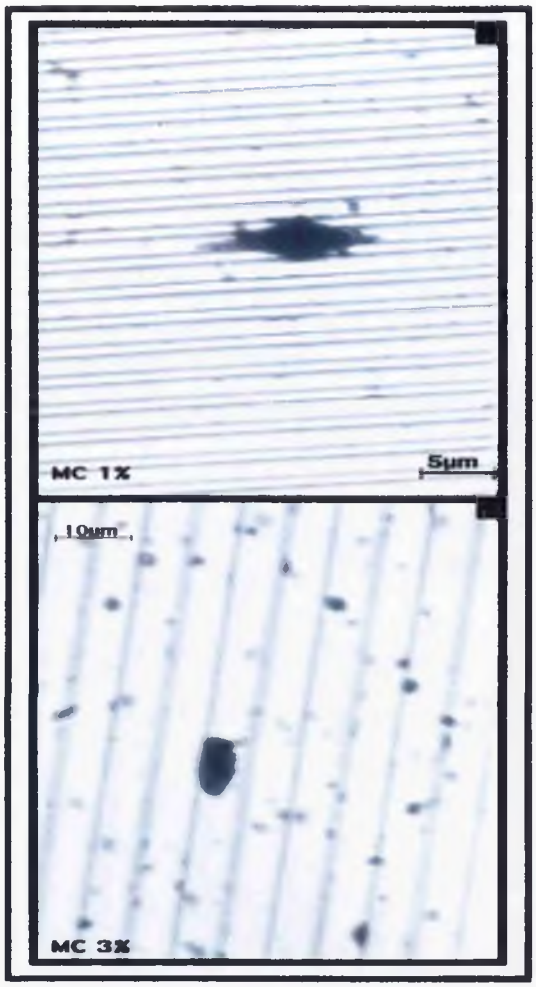


Figure 24:

Light microscopy images (20x-Objective) of Epitenon cells grown on quartz grooved topography with depth 5μm and 5μm width in 0.5% Polyvinylpyrrolidone and Control ECT media for 24h.
Fixed and stained with Coomassie blue

2 Control

3 PVP_1 with mol.wt.40,000

4 PVP_2 with mol.wt.360,000

Scale bars for all images 5 μm

Number above scale bar indicates groove width.

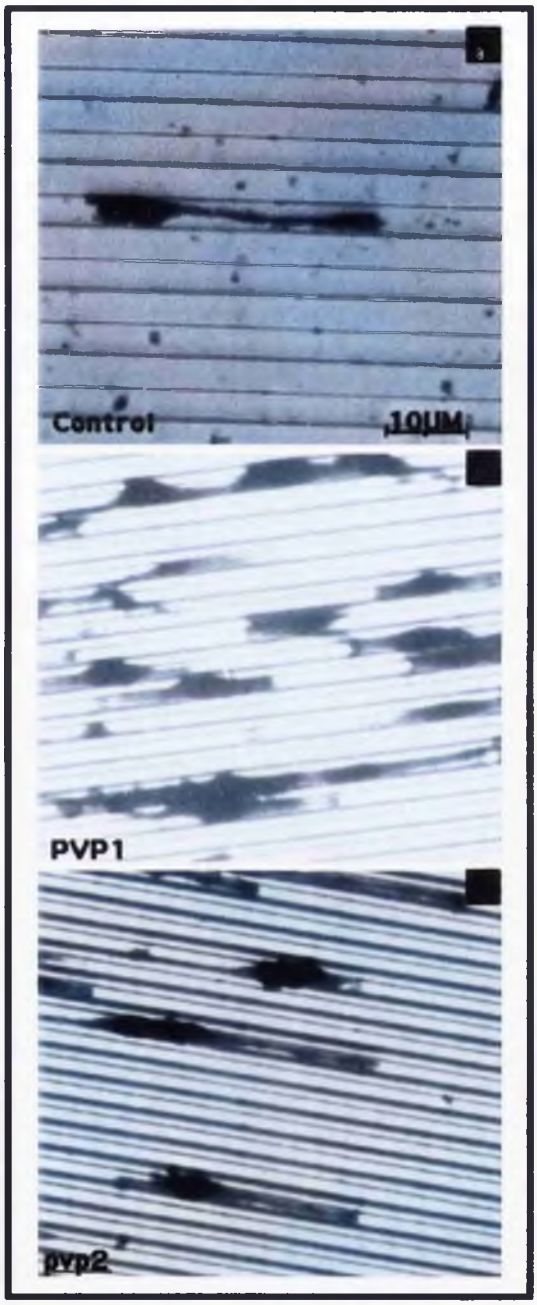


Figure 25:

Light microscopy images (20x-Objective) of Epitenon cells grown on quartz grooved topography with depth 5μm and 5μm width in Dextran with mol.wt 9,000 and Control ECT media for 24h.

Fixed and stained with Coomassie blue

1. Control
2. Dextran with conc.2%
3. Dextran conc. 4%.

Scale bars for all images 5 μm

Number above scale bar indicates groove width.

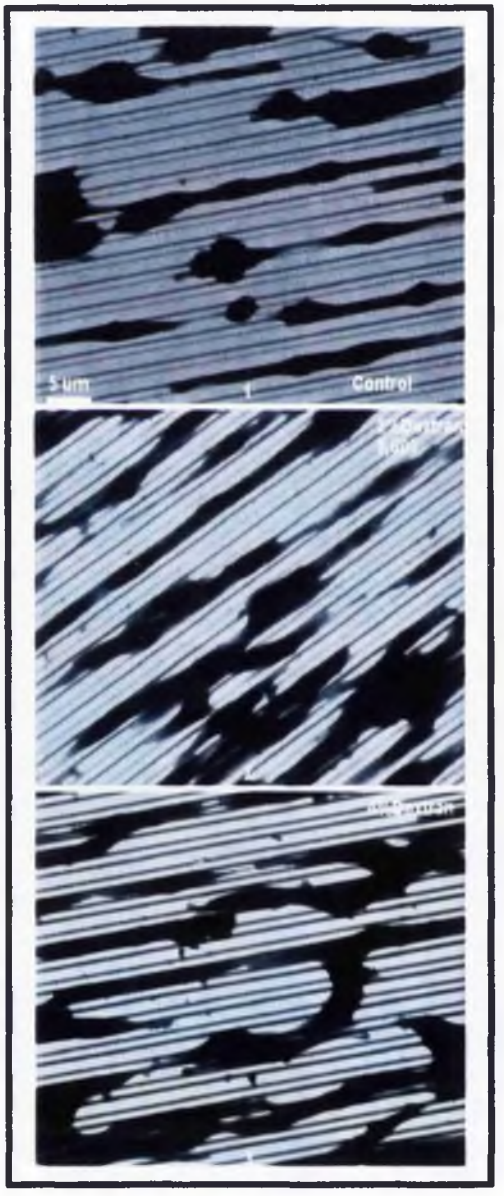


Figure 26:

Light microscopy images (20x-Objective) of Epitenon cells grown on quartz groove topography with depth 5μm and 5-10μm width in Ficoll with mol.wt 70,000 and Control ECT media for 24h. Fixed and stained with Coomassie blue

1. Ficoll 0.5%.
1. Ficoll 2%.
2. Ficoll 4%

Scale bars for all images 5 μm

Number above scale bar indicates groove width.

Figure 27:

Light microscopy images (20x-Objective) of Endothelial cells B10D(2) grown in Dextran with mol.wt. 503,000 for 24h on quartz grooved topography with depth 5μm and 5μm width.

Fixed and stained with Coomassie blue:

1. Control (Ham's F10)
2. Dextran conc. 2%
3. Dextran conc. 4%

Scale bars=5μm

Number above scale bar indicates groove width.

Figure 28:

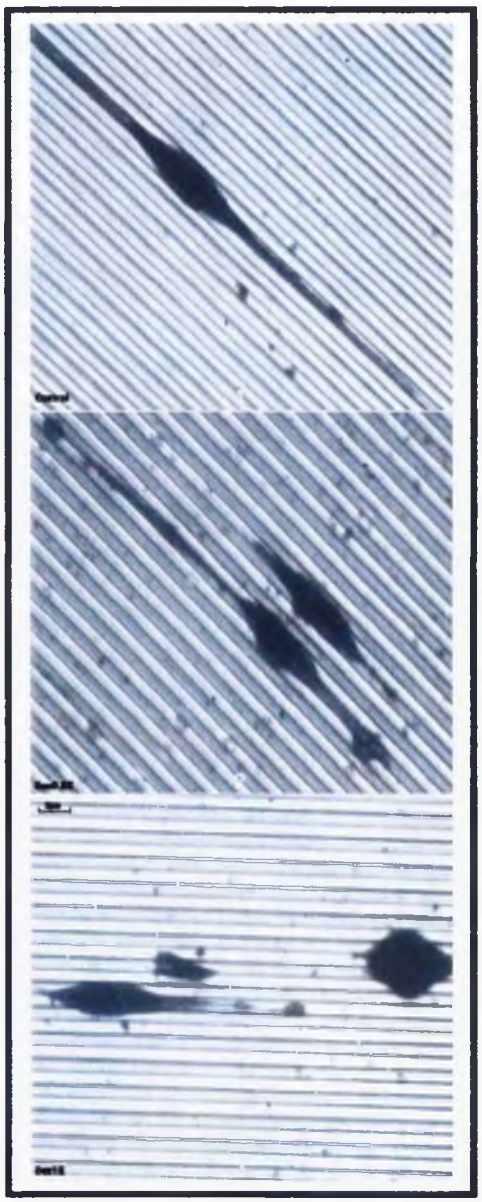
Light microscopy images (20x-Objective) of Endothelial cells B10D(2) grown in Dextran with mol.wt. 2000,000 for 24h on quartz grooved topography with depth 5μm and 5μm width.

Fixed and stained with Coomassie blue:

1. Control (Ham's F10)
2. Dextran conc. 0.5%
3. Dextran conc. 1%

Scale bars=5μm

Number above scale bar indicates groove width.



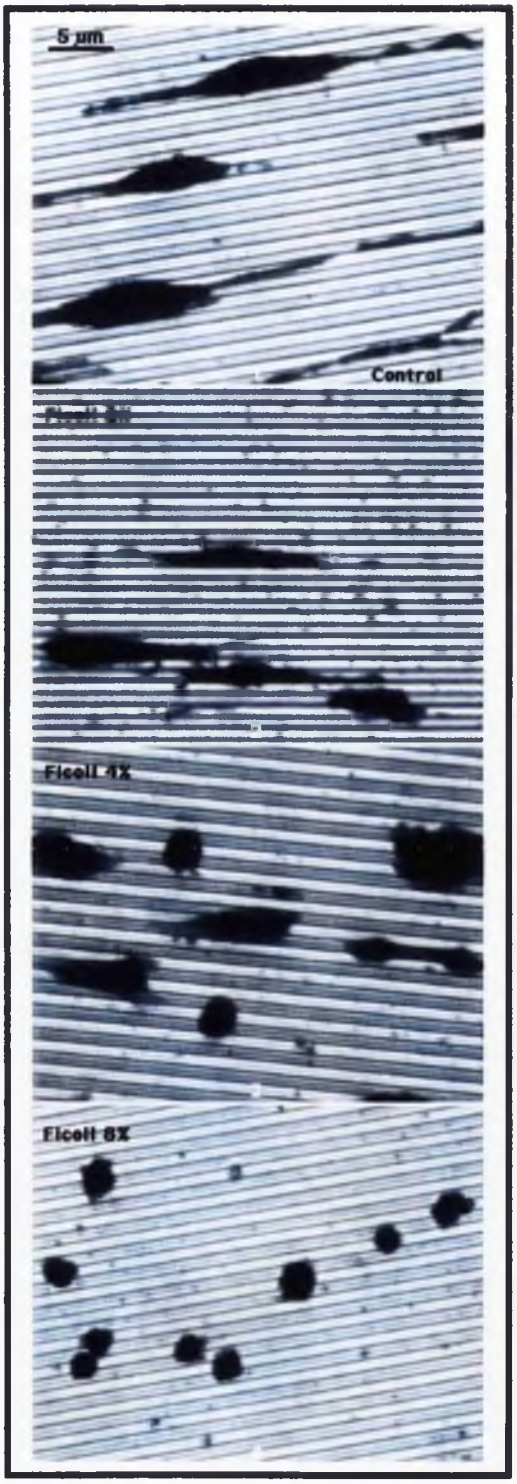


Figure 29:

Light microscopy images (20x-Objective) of Endothelial cells B10D (2) grown in Ficoll with mol.wt. 400,000 for 24h on quartz grooved topography with depth 5µm and 5µm width.

Fixed and stained with Coomassie blue:

1. Control (Ham's F10)
2. Ficoll conc.2%
3. Ficoll conc.4%
4. Ficoll conc.8%.

Scale bars for all images 5 µm

Number above scale bar indicates groove width.

3. 3. A study of the morphology of Endothelial cells grown in highly viscous media on planar topography as a control

3. 3. 1. The effect of Ficoll (mol.wt.400,000) on endothelial cell length:

It was found that the length of the Endothelial cells (B10D2) was affected by Ficoll concentration when cells were grown on plane topography as follows:

Table 12: Ficoll effect on endothelial cells on plane topography :

Ficoll Conc.	Standard deviation.	Average length (μm) Of 50 cells	P-value
Ham's F10	38.9	100	
Ficoll 4%	37.8	55.9	0.883
Ficoll 8%	1.8	10.3	<0.0001

This indicates that the length of the endothelial cells decrease with the increasing of Ficoll concentration as show in the Figure 31.

Figure 30:

The curve shows the decrease of endothelial length average with increase of medium viscosity. Standard deviation of the regression coefficient *(sb)* = - 0.961 and t = 22.3 for all Ficoll 400,000 results.

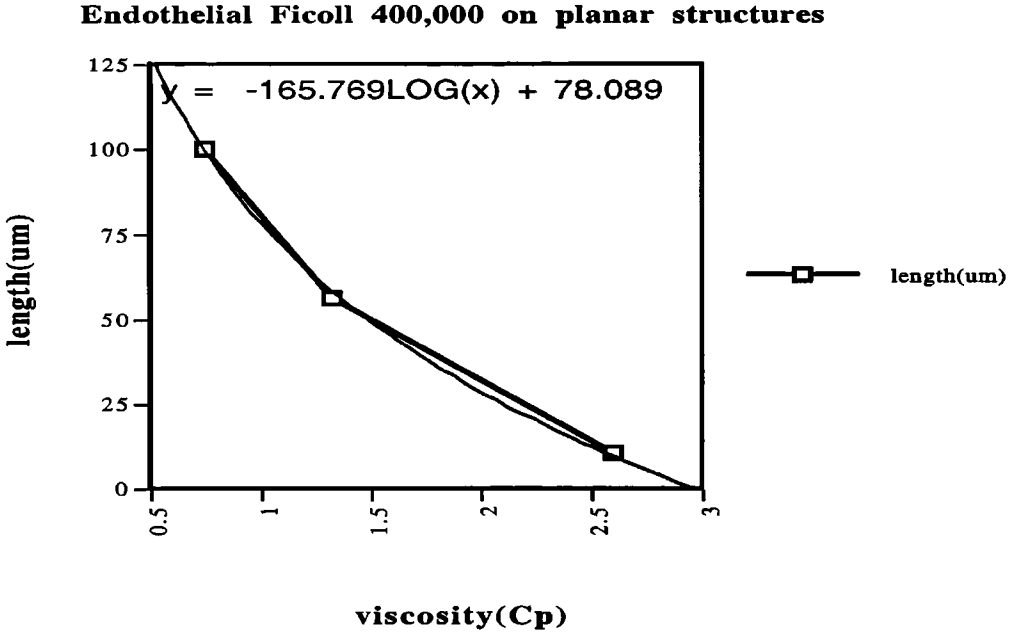
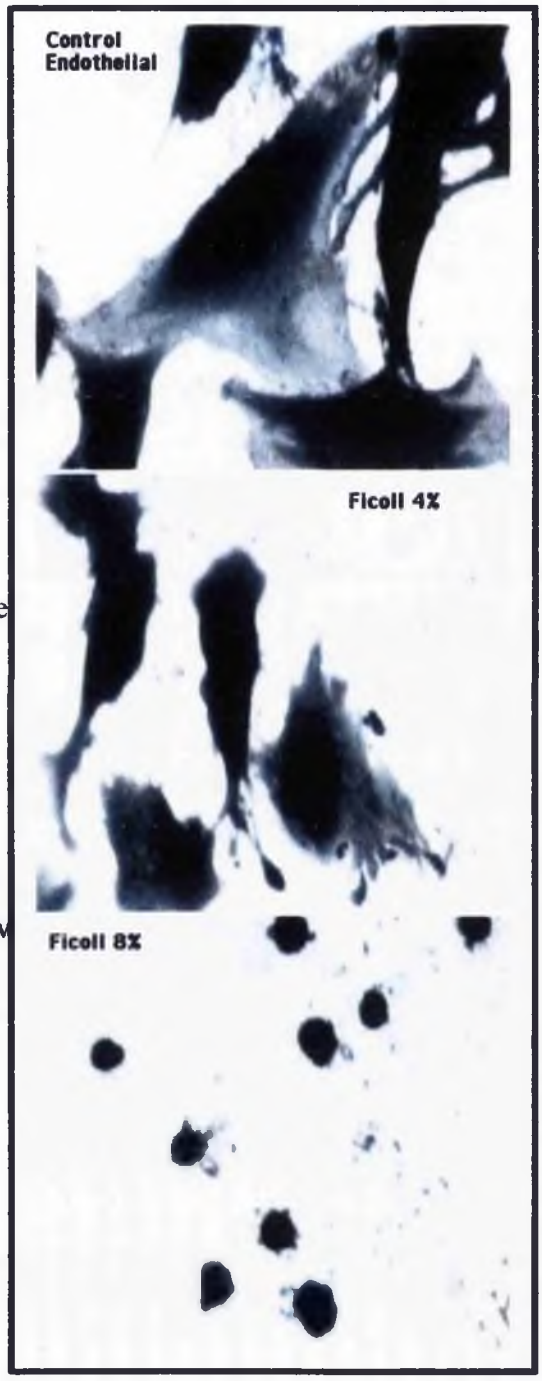


Figure 31:

Light microscopy images (20x-Objective) of Endothelial cells B10D2 grown on glass plane cover-slip in Ficoll 400,000 and control Ham's F10. Fixed and stained with Coomassie blue.

1. Control.
2. In Ficoll 4%.
3. In Ficoll 8%



It is clearly observed that there is a difference between control and experimental cells: The control cells appears spread with well developed lamellipodia. While the cells were grown in 4% Ficoll appears have some ability to spread and grow, cells were grown in Ficoll 8% appears lose their ability to grow or spread to the substratum.

Scale bars for all images = 5 μm
Number above scale bar indicates groove width.

Discussion and conclusion:

Comparison of the pictures suggests ways that viscosity could determine cell shape. Cell length decreased in medium with high viscosity see for example picture 3 & 4 in Fig 21 and picture b & c in Fig 22 for Epitenon cells and pictures 3 in Fig 28 and pictures 3 & 4 in fig 29 for Endothelial cells.

But changes in the morphological reactions occurred less frequently when cells were cultured in low molecular weight Dextran 9,000 or Ficoll 7000 with low and high concentrations, see the pictures in Fig 25 & 26. Quantitative data on the morphometry of cells exposed to these reagents are shown in pages 70 -77 and page 90.

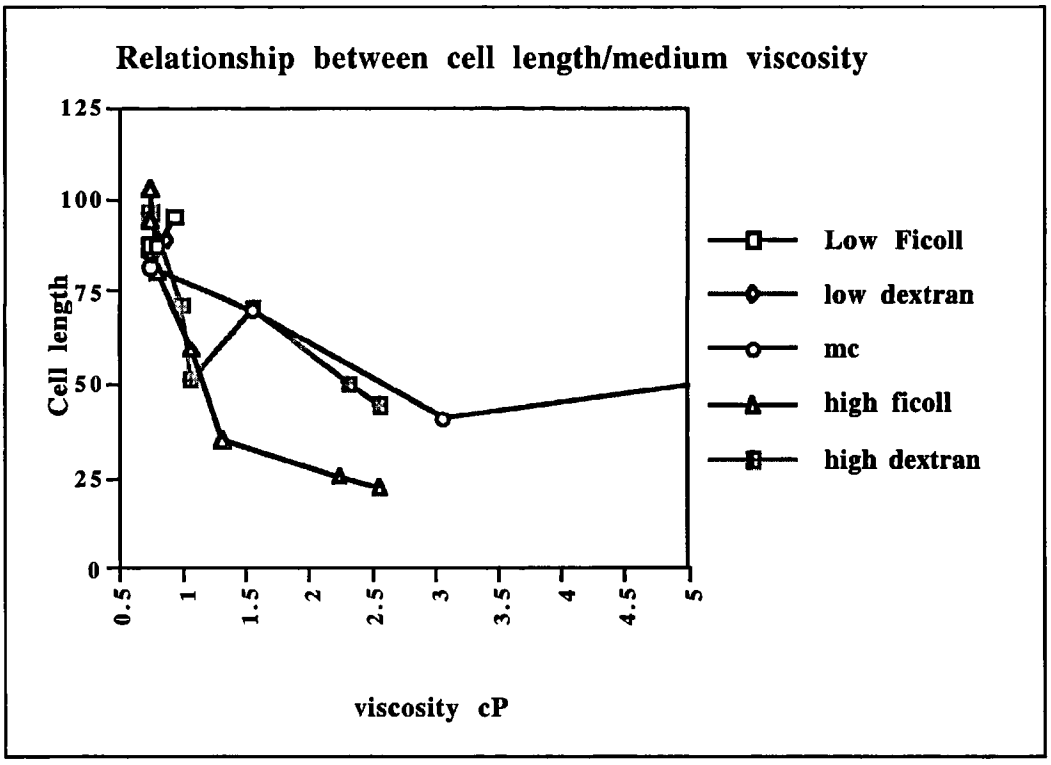


Figure 32:

The curves indicate that cell length decreases when the viscosity of the medium increases. The standard deviation of the regression coefficient ranged between 0.78 to 0.961). Comparing this to the length of the cells grown in low molecular weight reagents (low dextran standard deviation of the regression coefficient = - 2.687 and low ficoll Standard deviation of the regression coefficient = 0.900).

It is reasonable to assume that diffusion is being reduced when the viscosity is raised. The relation between viscosity and diffusion can be obtained from the Stokes-Einstein equation:

Equation 3: $D = kT / 6\pi\eta r$

Where D is diffusion coefficient, T is temperature, r is the radius of an ion, η is Coefficient of the viscosity by c.P. and k is Boltzmann constant=1.38066.

The next Table list the t-values testing the significance of standard deviation of the regression coefficient of the cell length (sb) against the regression coefficient of the viscosity (b)

High Dextran	High Ficoll	Methyl cellulose	Low Dextran	Low Ficoll	High Ficoll	High Dextran	High Ficoll
Epitenon cells on grooved topography					Endothelial cells		
					Groove		plane
25.2	53.1	3.6	0.05	43.6	90.7	53.1	22.3

Diffusion in turn may affect the topographical reaction of cells.
Most of cells became less spread when grown in highly viscous medium (as shown in the curve figure 32 that summarises these results).

Of special interest is the finding that cells are still alive after incubated in high viscosity medium as obtained from the recovery experiments, see Fig 9 & 10.
From the results I conclude that the medium viscosity affects the cell length and width (in very highly viscous medium).
The viscosity in turns affects diffusion around cells, and therefore may affect the morphology of the cells.
My study indicates that the reaction to topography is reduced as the medium viscosity is increased for the high concentration of all reagents used in the experiment and that can be tested by further experiments.
The next stage of the study is the using of Interference Reflection Microscopy (IRM). See next section.

4. THE INTERFERENCE REFLECTION MICROSCOPE (IRM) STUDY

Interference reflection microscopy, was used in these experiments to study the adhesion of epitenon and endothelial cells to plane and groove structures in control media and experimental media of high viscosity.

Using this technique we can measure or at least estimate the separation distances between cells and substrate surfaces. This is the region between cell and substratum where diffusion must occur. To calibrate IRM correctly the refractive indices of the media were measured, (see equation below).

Thus IRM can be used to reveal the closeness of approach of the cell to the substrate and thus the accessibility of this region to entry and emigration of solutes by diffusion.

Additionally, using IRM methods, I can test the hypothesis that cells do not conform to many topographies as closely as they would to planar surfaces.

IRM images analysis

The IRM micrographs are patterns made out of destructive and constructive interfering reflections. The intensity of reflected light, after interference, has been described by (Curtis, 1964) and (Vasicek, 1960). This is given by the next equation:

Equation 4:

$$p = \frac{n_1^2(n-n_0)^2\cos^2(\frac{\pi}{\lambda}2n_1 d\cos\theta) + (n_1^2 - n_0 n)^2\sin^2(\frac{\pi}{\lambda}2n_1 d\cos\theta)}{n_1^2(n+n_0)^2\cos^2(\frac{\pi}{\lambda}2n_1 d\cos\theta) + (n_1^2 + n_0 n)^2\sin^2(\frac{\pi}{\lambda}2n_1 d\cos\theta)}$$

That was used to calculate the reflectivity ratio (p)

Therefore n(cell)=1.37, n_0 (substrate)=1.341, n_1 (medium)=1.34 -1.352, wavelength (λ)[m]=5.46E-07,p (back)=0.00356 and Cross over nm=48

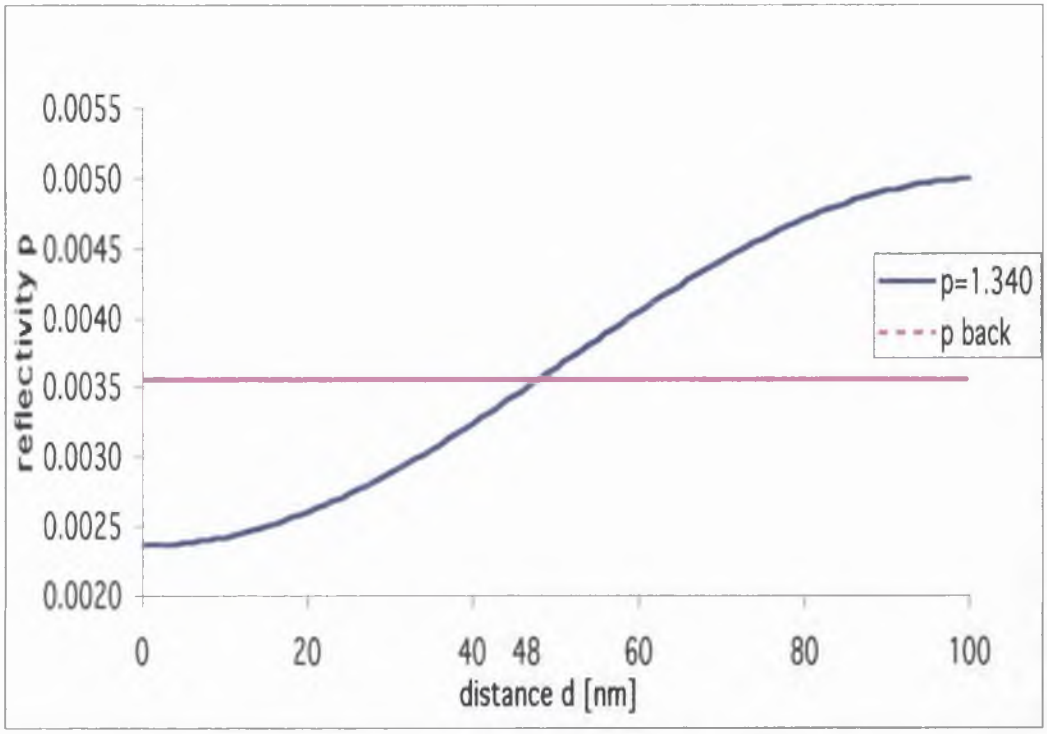


Figure 33:

Graph of reflectivity ratio against gap-distance.

4.1. REFRACTIVE INDEX MEASUREMENTS

It is important to measure the refractive index of the medium when doing any IRM study because the expected IRM image reflectivity responds to changes in n_1 (the refractive index of the medium).

The aim of these measurements are to measure the physical properties of the chemical compounds, (viscosity enhancing reagents used in the first and second section of the Results) to provide basic data for calculating the expected background reflections in IRM and the Interference microscope.
The refractive index for all reagents with at given concentrations was measured at two different temperature (25° C and 37° C), the results are shown in Table 13.

Theory:

When a ray of light passes from one substance to another its velocity may change. This phenomenon is called refraction and the magnitude of the effect is measured in terms of the refractive index (η). The effect is related to the velocity of light in the substance and the refractive index is defined by Snell's law:

Equation 5:

$$\eta = \sin i / \sin r = v / v'$$

η is the index of refraction, i is the angle of incidence, r the angle of refraction, v the velocity of light in the first medium, v' the velocity of light in the second medium

Results:

The measurements results indicate that there is a significant difference between the refractive indices of the reagents tested in the experiment, which was measured with an Abbé refractometer. The values ranged between 1.341 and 1.352. As shown in the Table (13).

Table13: The refractive index measurements:

Reagent's name &conc.	η at 25° C	η at 37° C
ECT	1.342	1.340
Dextran 9,000 conc.0.5%	1.343	1.341
Dextran 9,000 conc.2%	1.345	1.343
Dextran 9,000 conc.4%	1.347	1.345
Dextran 503,000 conc.1%	1.343	1.341
Dextran 503,000 conc.2%	1.345	1.343
Dextran 503,000 conc.4%	1.348	1.347
Dextran 2000,000conc.0.5%	1.343	1.341
Dextran 2000,000 conc.2%	1.345	1.343
0.5%Polyvinylpyrrlidone 40,000	1.343	1.342
0.5%Polyvinylpyrrlidone 360,000	1.343	1.342
Carboxymethylcellulose 0.5%	1.343	1.342
Methylcellulose 0.5%	1.342	1.341
Methylcellulose 1%	1.343	1.342
Methylcellulose 2%	1.344	1.343
Methylcellulose 3%	1.346	1.345
Ficoll 70,000 conc.0.5%	1.344	1.343
Ficoll 70,000 conc.2%	1.345	1.344
Ficoll 70,000 conc.4%	1.346	1.345
Ficoll 400,000 conc.0.5%	1.343	1.341
Ficoll 400,000 conc.2%	1.345	1.343
Ficoll 400,000 conc.4%	1.347	1.345
Ficoll 400,000 conc.6%	1.350	1.349
Ficoll 400,000 conc.8%	1.352	1.351

4.2 Study the effects of highly viscous medium on Epitenon cells using IRM.

The IRM results

As shown in the Figure (35-58). I obtained the following results:
1-The cultures grown in low molecular weight reagents shows no significant image difference between cells grown for 24hrs and those cells that were grown for only 3hrs in Dextran 9,000 for all the given concentrations. There is also no significant difference between them and the control cells (ECT media for epitenon and Ham's F10 media for endothelial).
Cells grown in Ficoll 70,000 gave similar results.

2-The cultures grown in high molecular weight reagents (molwt.>100000) showed cells with a lot of dark regions when the viscosity of the medium increased.
In very high viscous media cells were rounded and not very well spread, low in number and very small in size with many fringes around them, the fringes are not colored. I investigated this more by measuring the black, dark and grey areas in the studied cells. And also by measuring the refractive index of all reagents at the given concentration.

The reflectivity ratio measurements for the background:

There are illustrated by the graph Figure (34). This graph shows that the minima are closer than 20nm and the linear range of the distance/reflectivity graph extends to ca.80nm.

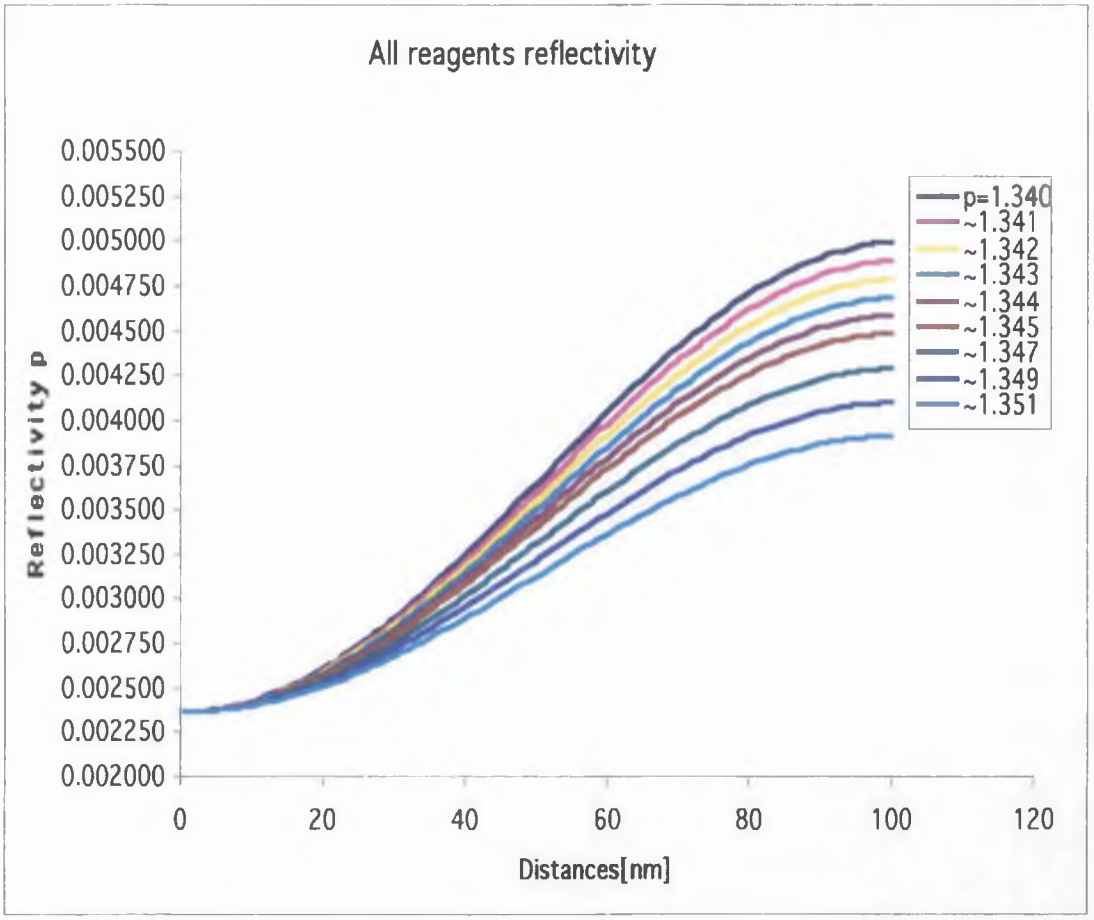


Figure 34:

Curves show the reflectivity ratio (p) against gap- distances (d) for cells grown in different media where n_0 is ranged from 1.340 to 1.351.

Epitenon IRM Results:

I-Planar topography:

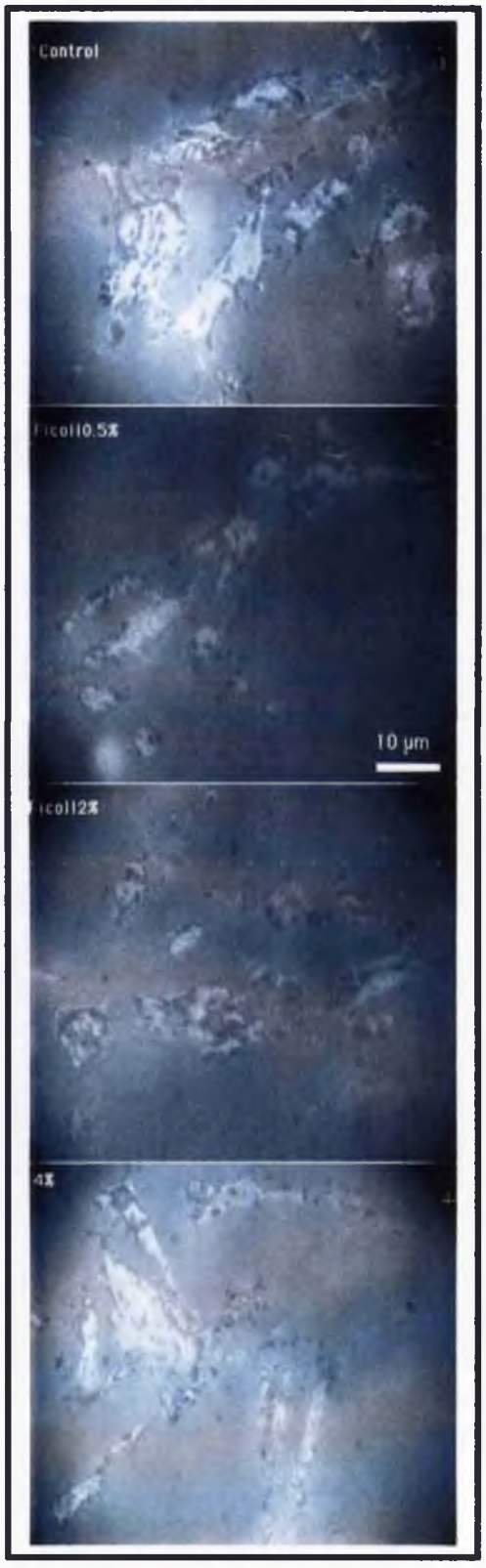


Figure 35:

IRM images (50x-objective) show epitenon cells were grown for 24h on plane glass cover-slip in Ficoll 70,000

1. Control
2. Conc.0.5%
3. Conc.2%
4. Conc.4%

Figure 36:

IRM images (50x-objective) show epitenon cells were grown for 24h on plane glass cover-slip in Ficoll400,000

1. Conc.0.5%
2. Conc.2%
3. Conc.6%
4. Conc.8%

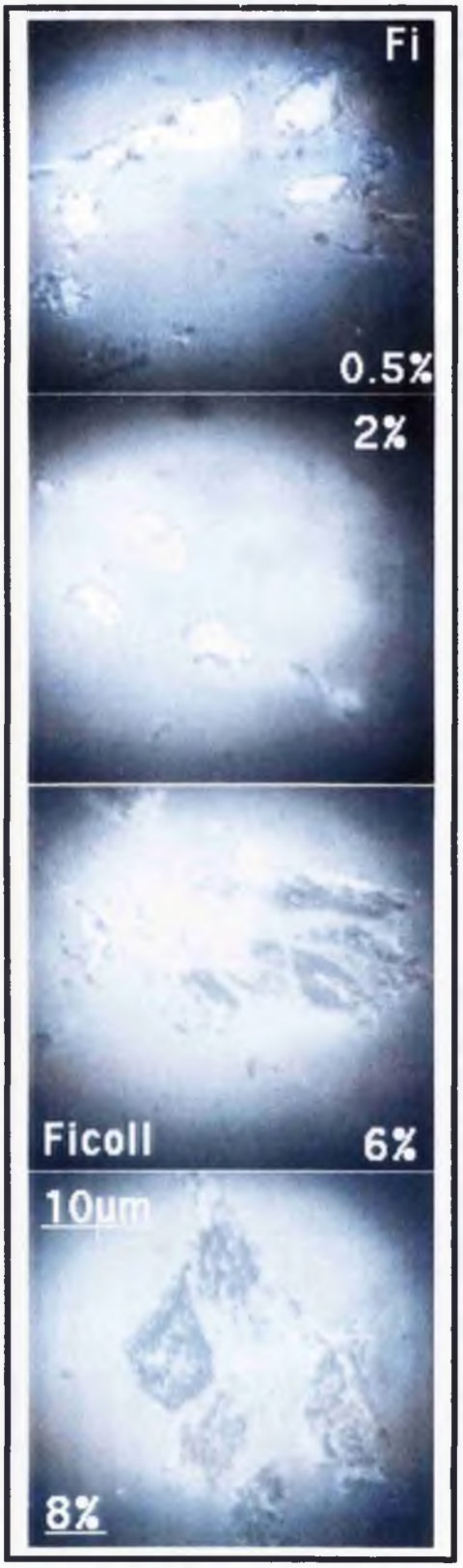


Figure 37:

IRM (50x-objective) pseudocolour images, show epitenon cells were grown for 24h on plane glass cover-slip in Ficoll 400,000

1. Control, dark regions (orange in image) indicates separation distances of 36nm from substratum and light regions (yellow in image) indicates separation distances of 55nm from substratum.

2. Ficoll 2%, dark regions (dark red in image) indicates separation distances of 51nm from substratum and light regions (red in image) indicates separation distances of 60nm from substratum.

3. Ficoll 6%, dark regions (orange in image) indicates separation distances of 43nm from substratum and light regions (yellow in image) indicates separation distances of 50nm from substratum.

4. Ficoll 8%, dark regions (orange in image) indicates separation distances of 38nm from substratum and light regions (yellow in image) indicates separation distances of 52nm from substratum.

Bars 10 μm.

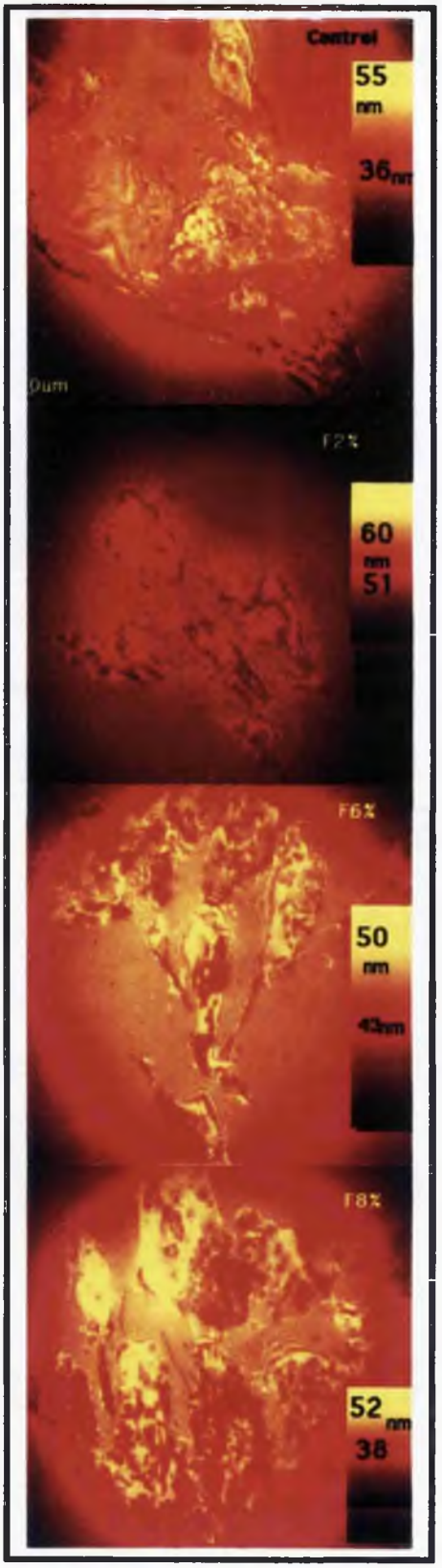


Figure 38:

IRM (50x-objective) pseudocolour images, show epitenon cells were grown for 3h on plane glass cover-slip in Ficoll 400,000

1. Control, dark regions (dark green in image) indicates separation distances of 49nm from substratum and light regions (green in image) indicates separation distances of 56nm from substratum.
2. Ficoll 0.5%, dark regions (dark blue in image) indicates separation distances of 53nm from substratum and light regions (blue in image) indicates separation distances of 56nm from substratum.
3. Ficoll 4%, dark regions (dark blue in image) indicates separation distances of 49nm from substratum and light regions (blue in image) indicates separation distances of 58nm from substratum.
4. Ficoll 8%, dark regions (blue in image) indicates separation distances of 34nm from substratum and light regions (green in image) indicates separation distances of 41nm from substratum.

Bars 10 μm

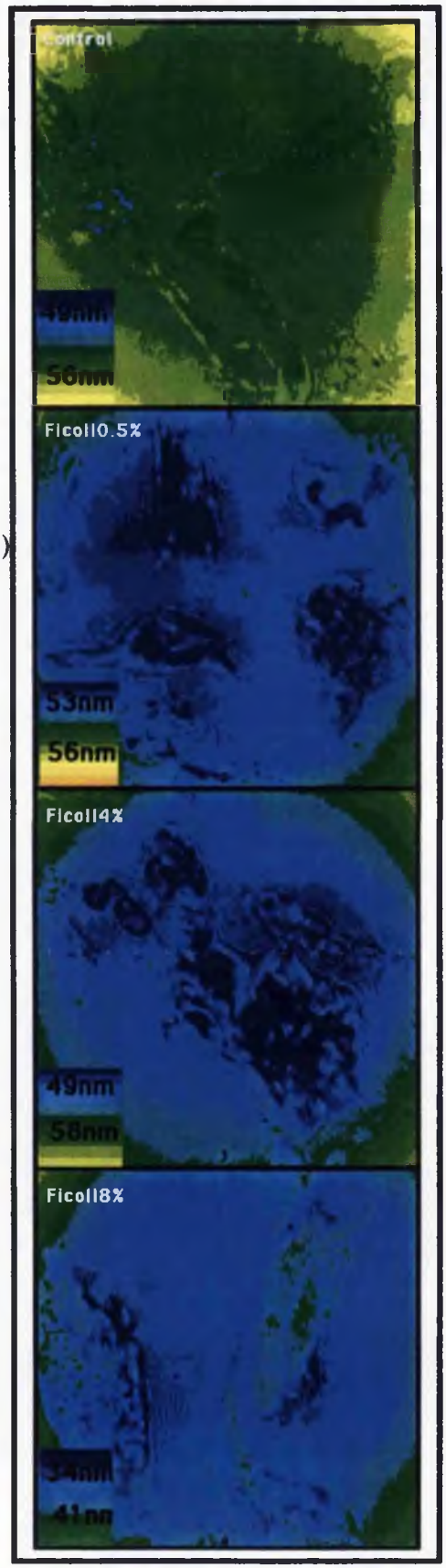


Figure 39:

Contour map (isopach) of IRM image (50x-objective) of Epitenon cells grown on plane quartz in 0.5% Ficoll400, 000 for 24h, Bars 10 μm: Threshold 80 = 48nm. White colour areas (bright regions) indicates separation distance (further away than the value in nm marked on the image) and the measurements in grey area (dark regions) indicates separation distance (closer than the value in nm marked on the image), the map shows that the light regions are more prominent in this cell. Data shows that the bright regions > 57 to 63nm and the dark regions < 34 to 41nm.

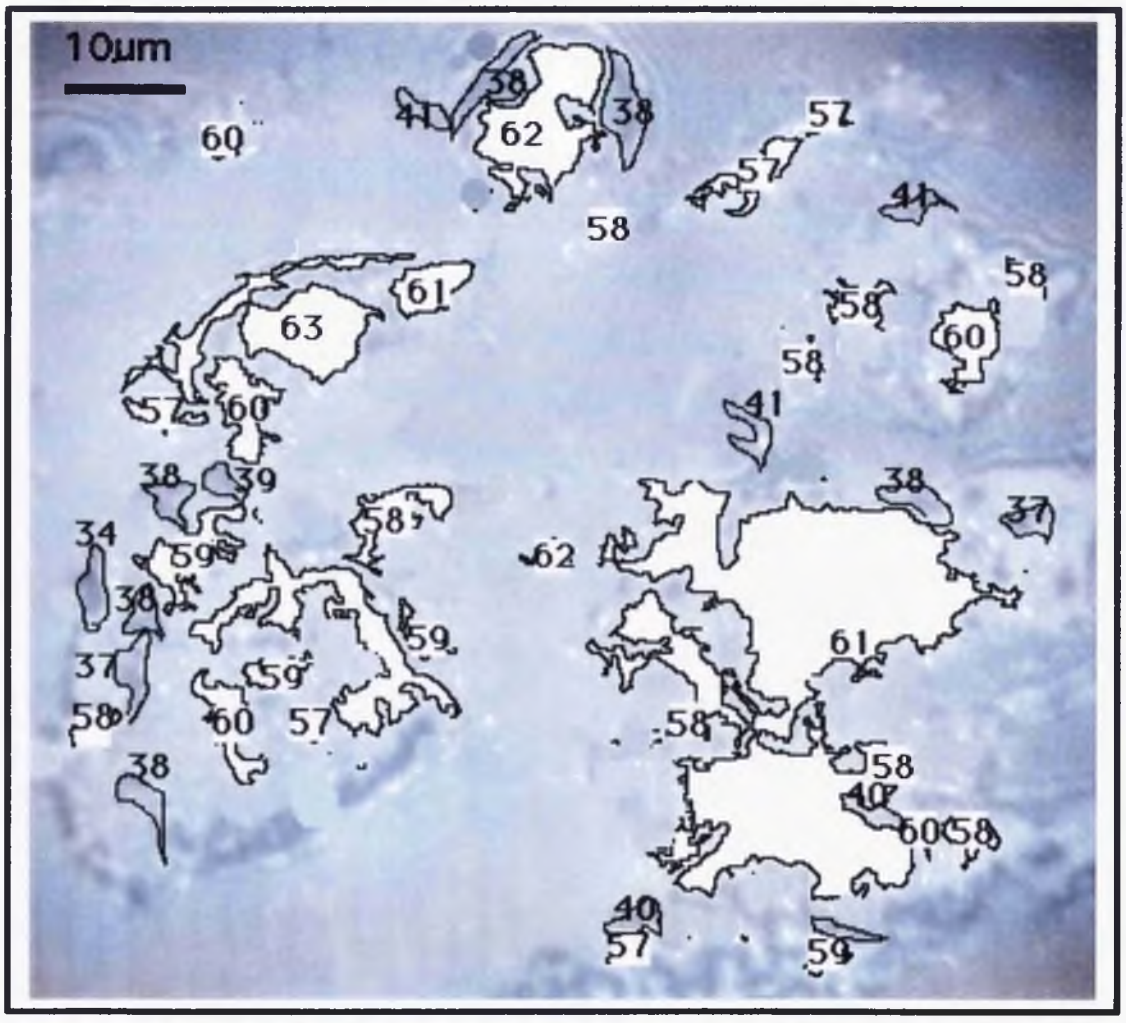


Figure 40:

Contour map (isopach) of IRM image (50x-objective) of Epitenon cells grown on plane quartz in 8% Ficoll 400,000 for 24h. Bars 10 μm: Threshold 90 = 48nm. Image shows light regions (white colour) measurements (separation distance further away than the value in nm marked on the image). And dark regions measurements (separation distance closer than the value in nm marked on the image). While cells cultured in 8% Ficoll have fewer light regions than those cultured in 0.5% Ficoll, see the picture in Fig 37, the cell appeared with more dark regions in 8% Ficoll. Data shows that the bright regions > 55 to 61nm and the dark regions < 36 to 40nm.

II-Grooved topography:

Figure 41:

IRM images (50x-objective) for Epitenon cells that were grow in Dextran 503,000 for 24h on shallow groove quartz structures with 280-420nm depth and 10 μm width :

1 Control.

2 Dextran 1%.

3 Dextran 2%.

4 Dextran 4%.

Cells appeared smaller in size and have more dark regions when they were cultured in high molecular weight Dextran than control cells.

Bars 10μm

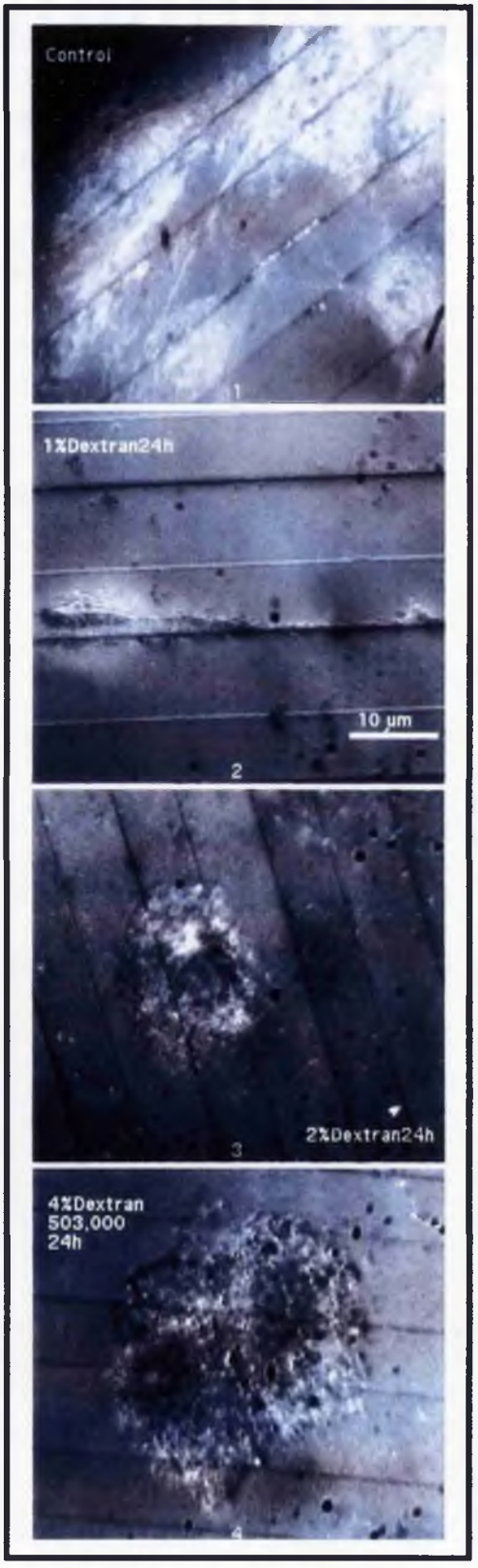


Figure 42:

IRM images (50x-objective) for Epitenon cells that were grow in Dextran 503,000 for 3h on shallow groove quartz structures with 280-420nm depth and 5-10 μm width :

1 Control.

2 Dextran 2%.

3 Dextran 4%.

Although cells were incubated for only 3h in Dextran with high molecular weight, they still appeared smaller in size and still have more dark regions than control cells.

Bars 10μm

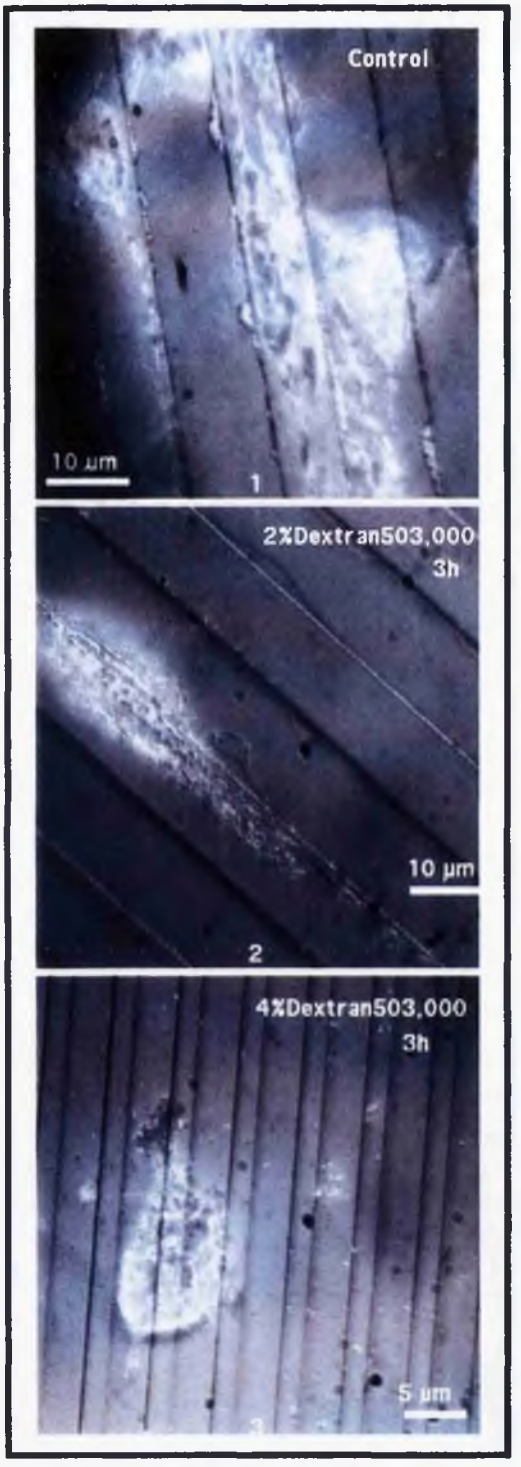


Figure 43:

IRM images (50x-objective) for Epitenon cells that were grow in Dextran 2000,000 for 24h on shallow groove quartz structures with 280-420nm depth and 10 μm width :

1 Control

2 Dextran 0.5%.

3 Dextran 2%.

There are significant differences between cells cultured in 2% Dextran 2000,000 and cell cultured in only 0.5%Dextran or control medium. Image of cells in high molecular weight Dextran became smaller in size and almost dark see picture 3.

Bars 10μm

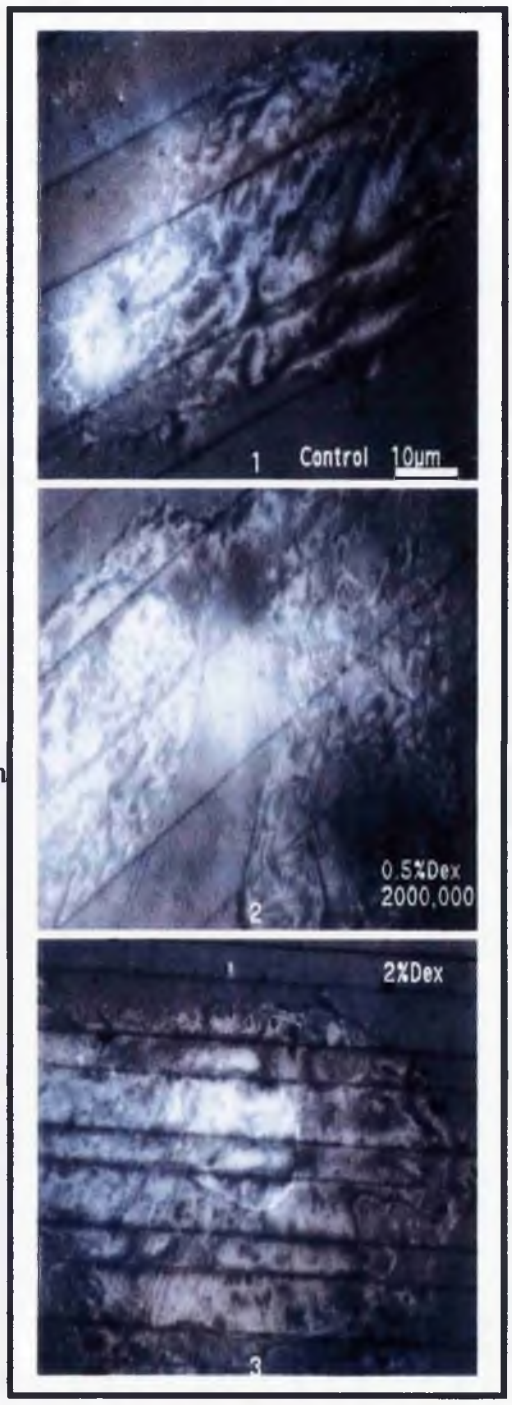
Figure 44:

IRM images (50x-objective) for Epitenon cells that were grow in Dextran 2000,000 for 3h on shallow groove quartz structures with 280-420nm depth and 5-10μm width :

1 Control

2 Dextran 0.5%.

3 Dextran 2%.



Although cells were incubated for 3h in high molecular weight Dextran , but still cells appeared normal in size in high concentration of Dextran see picture 2.

Bars 10μm

Figure 45:

IRM images (50x-objective) for Epitenon cells that were grow in Ficoll 400,000 for 24h on shallow groove quartz structures with 280-420nm depth and 10μm width :

1. Control.
2. Ficoll 0.5%.
3. Ficoll 4%.
4. Ficoll 8%.

The pictures indicate that cells decreased in size with increasing concentration of Ficoll (high molecular weight) in the medium, see last picture.

Bars 10μm

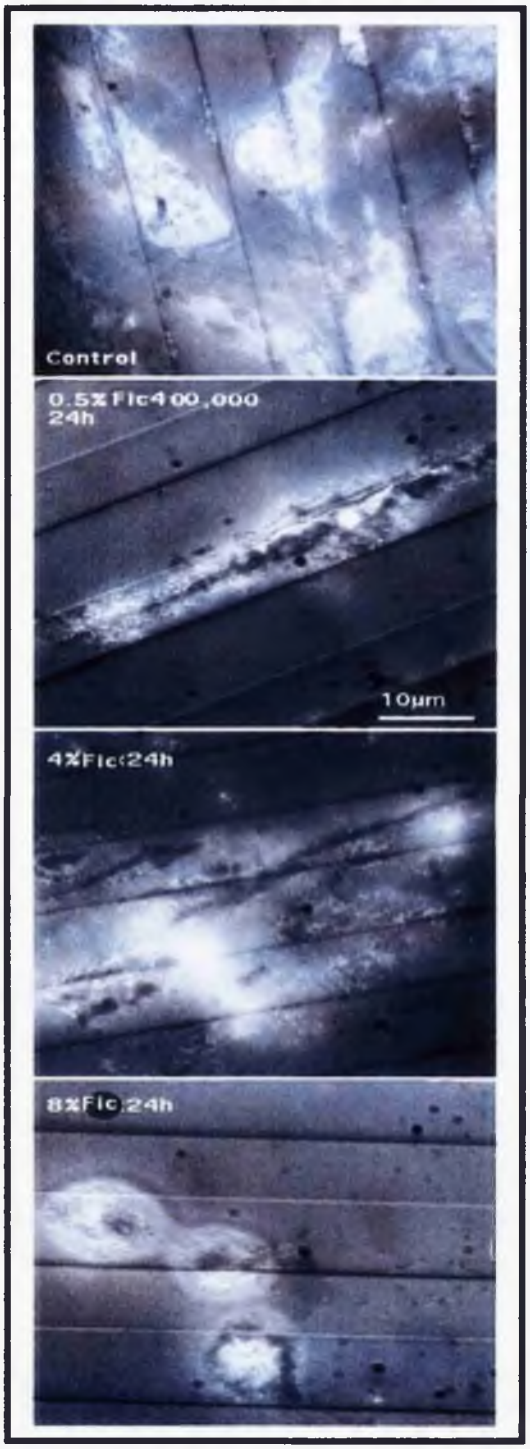
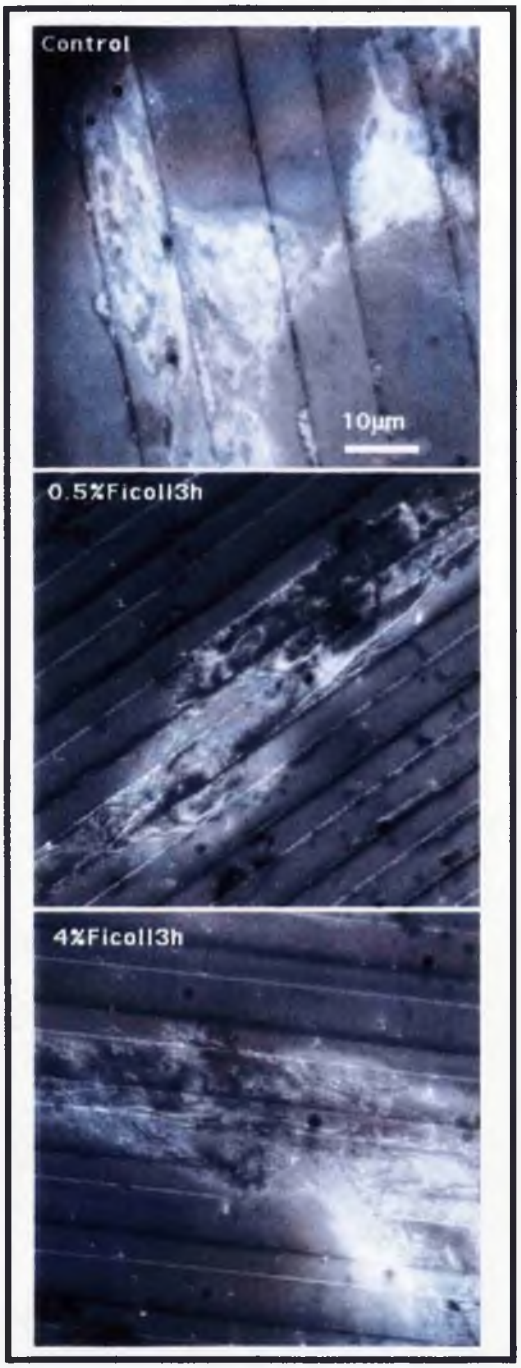


Figure 46:

IRM images (50x-objective) for Epitenon cells that were grow in Ficoll 400,000 for 3h on shallow groove quartz structures with 280-420nm depth and 5-10μm width :

1. Control.
2. Ficoll 0.5%.
3. Ficoll 4%.



No clear differences between cells cultured for only 3h in control medium or cells cultured in low or high concentration of Ficoll 400,000 see 3 pictures.

Bars 10μm

Endothelial Results:

I- Planar topography

Figure 47:

IRM pseudocolour images (50x-objective) for Endothelial B10D(2) cells grown in Dextran 503,000 for 24h on plane glass cover-slip:

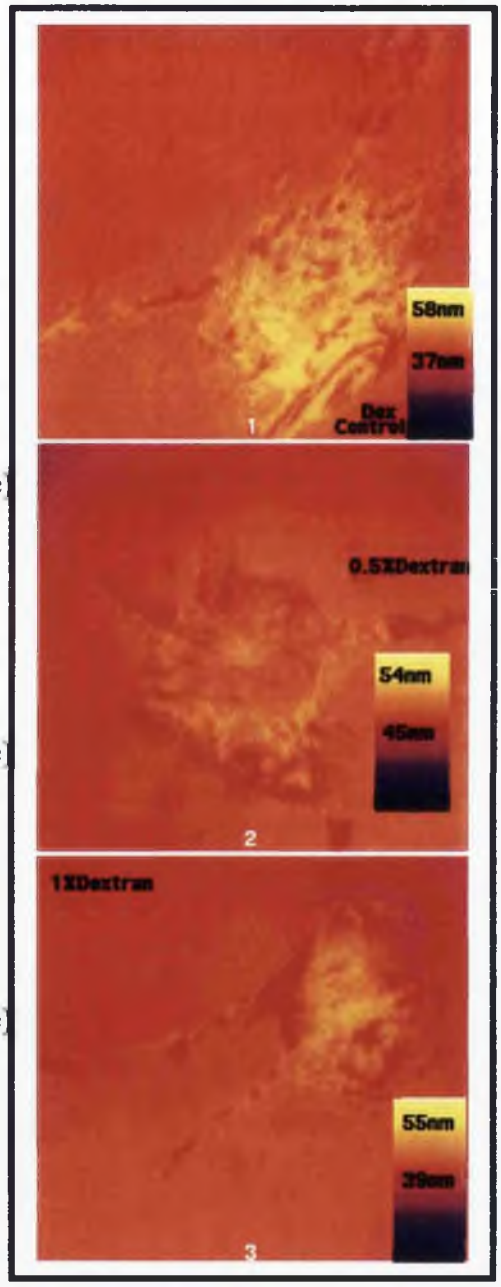
1. Control
2. Dextran 2%
3. Dextran 4%

Bars 10 µm.

Note that the control cell appeared well spread with highly developed lamellipodia comparing with experimental cells.

Figure 48:

IRM pseudocolour images (50x-objective) for Endothelial B10D(2) cells grown in Dextran 2000.000 for 24h on plane glass cover-slip:



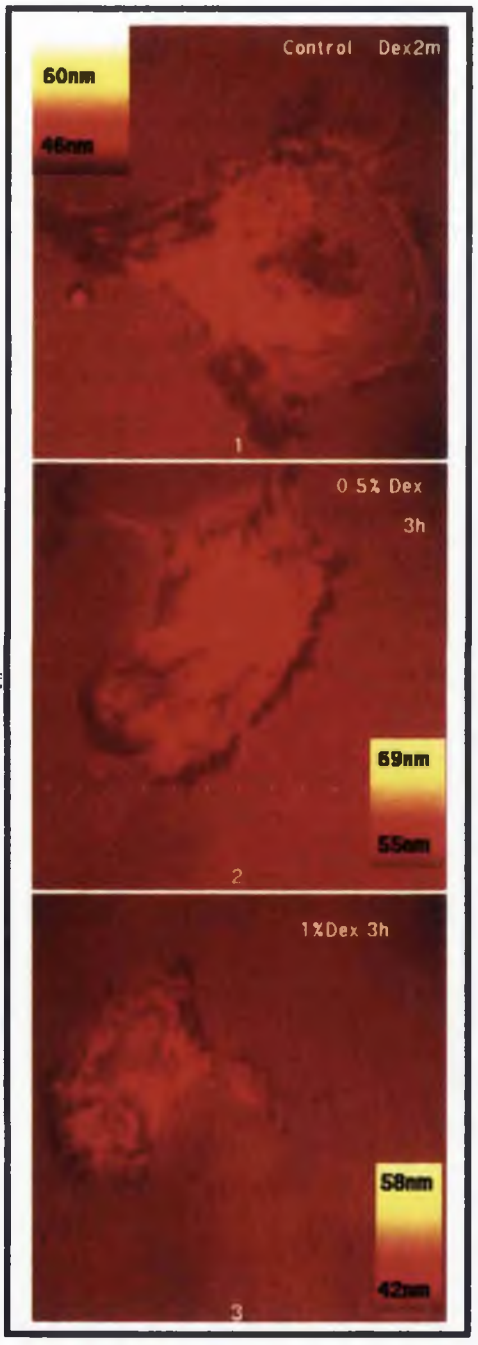
1. Control, dark regions (red in image) indicates separation distances of 37nm from substratum and light regions (orange in image) indicates separation distances of 58nm from substratum.
2. Dextran 0.5%, dark regions (red in image) indicates separation distances of 45nm from substratum and light regions (orange in image) indicates separation distances of 54nm from substratum.
3. Dextran 1%, dark regions (red in image) indicates separation distances of 39nm from substratum and light regions (orange in image) indicates separation distances of 55nm from substratum. Cells in high Dextran appeared smaller and darker than control cells.

These results indicate that the separation distances decreased when the medium viscosity increased.

Bars 10 μm.

Figure 49:

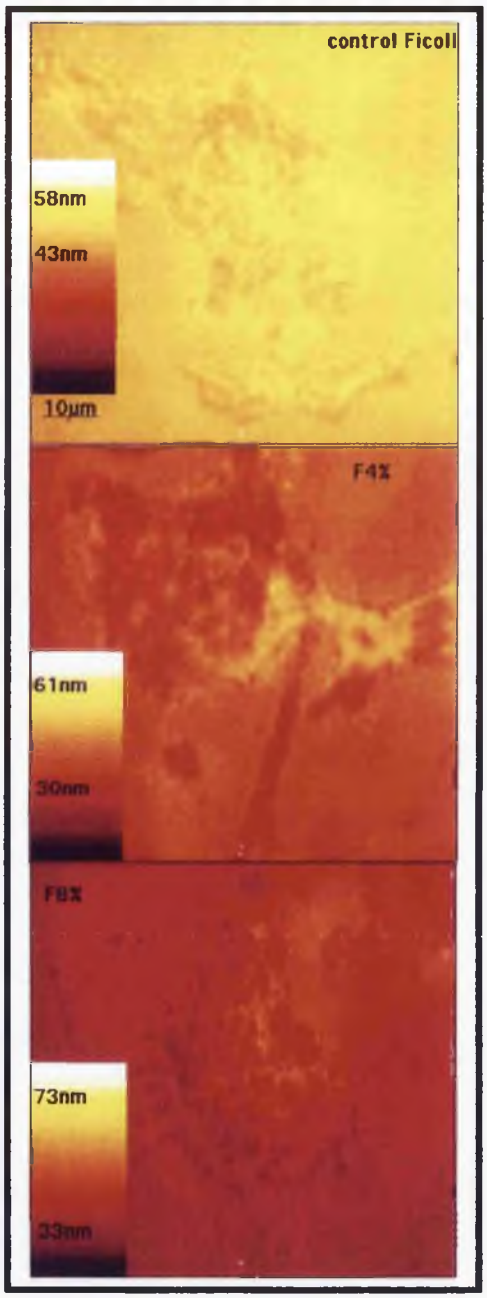
IRM pseudocolour images (50x-objective) for Endothelial B10D(2) cells grown in Dextran 2000,000 for 3h on plane glass cover-slip:



1. Control, dark regions (dark red in image) indicates separation distances of 46nm from substratum and light regions (red in image) indicates separation distances of 60nm from substratum.
2. Dextran 0.5%, dark regions (dark red in image) indicates separation distances of 55nm from substratum and light regions (red in image) indicates separation distances of 69nm from substratum.
3. Dextran 1%, dark regions (dark red in image) indicates separation distances of 42nm from substratum and light regions (red in image) indicates separation distances of 58nm from substratum. Cells appeared smaller and darker in high Dextran than in control medium.

Bars 10 μm.

Figure 50:



IRM pseudocolour images (50x-objective) for Endothelial B10D(2) cells grown in Ficoll 400,000 for 24h on plane glass cover-slip:

1. Control, dark regions (orange in image) indicates separation distances of 43nm from substratum and light regions (yellow in image) indicates separation distances of 58nm from substratum.

2. Ficoll 4%, dark regions (orange in image) indicates separation distances of 30nm from substratum and light regions (yellow in image) indicates separation distances of 61nm from substratum.

3. Ficoll 8%, dark regions (orange in image) indicates separation distances of 33nm from substratum and light regions (yellow in image) indicates separation distances of 73nm from substratum.

Bars 10 µm.

Data analysis:

All separation distances measurements were obtained using the computer programs (NIH) and Excel analysis to measure grey level and calculate the separation distances.

Figure 51:

Contour map (isopach) of IRM image (50x-objective) for Endothelial cell B10D(2) cultured in Ham's F10 medium as a control for cells were grown in 4%Dextran 503,000, bars 10μm.

Cell appeared with very large area of light regions indicated by white colour where are the separation distances are further away than the value in nm marked on the image, they ranged between > 55 - 76nm. The dark areas indicate dark regions where are the separation distances are closer than the value in nm marked on the image, ranged < 37 - 53nm.

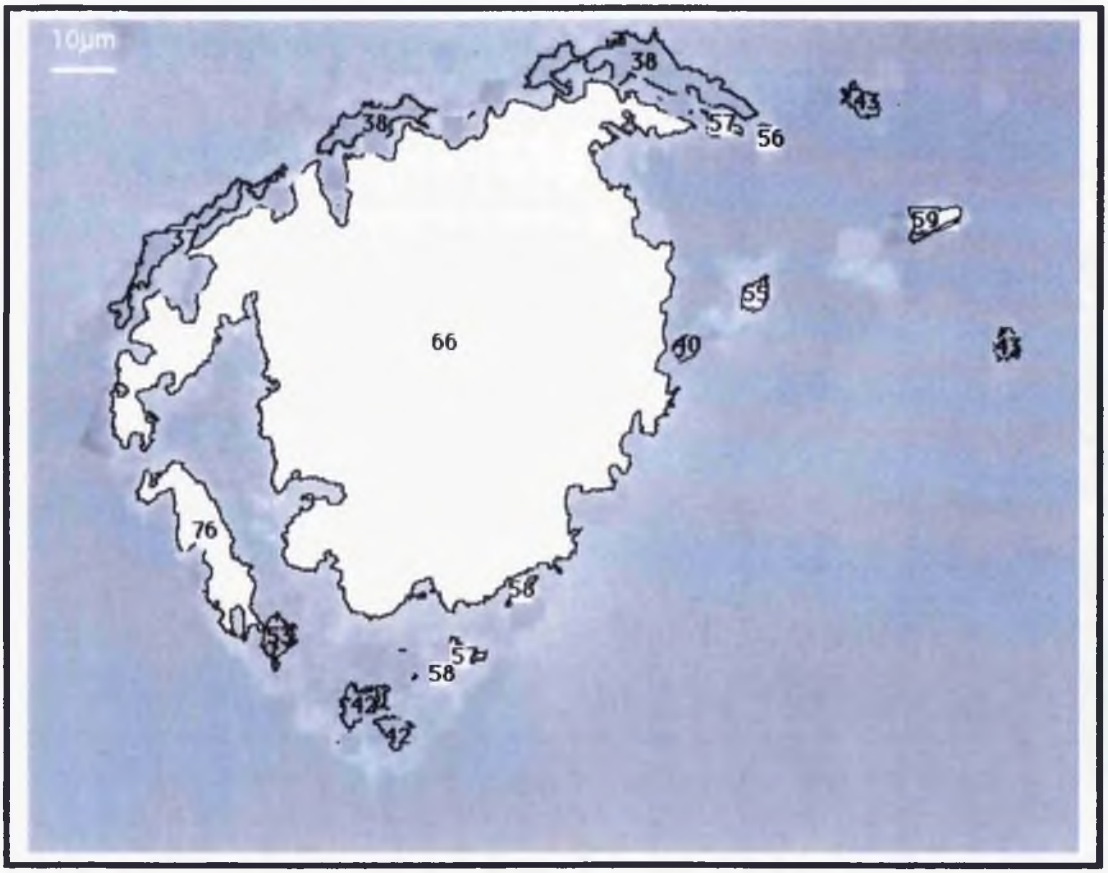
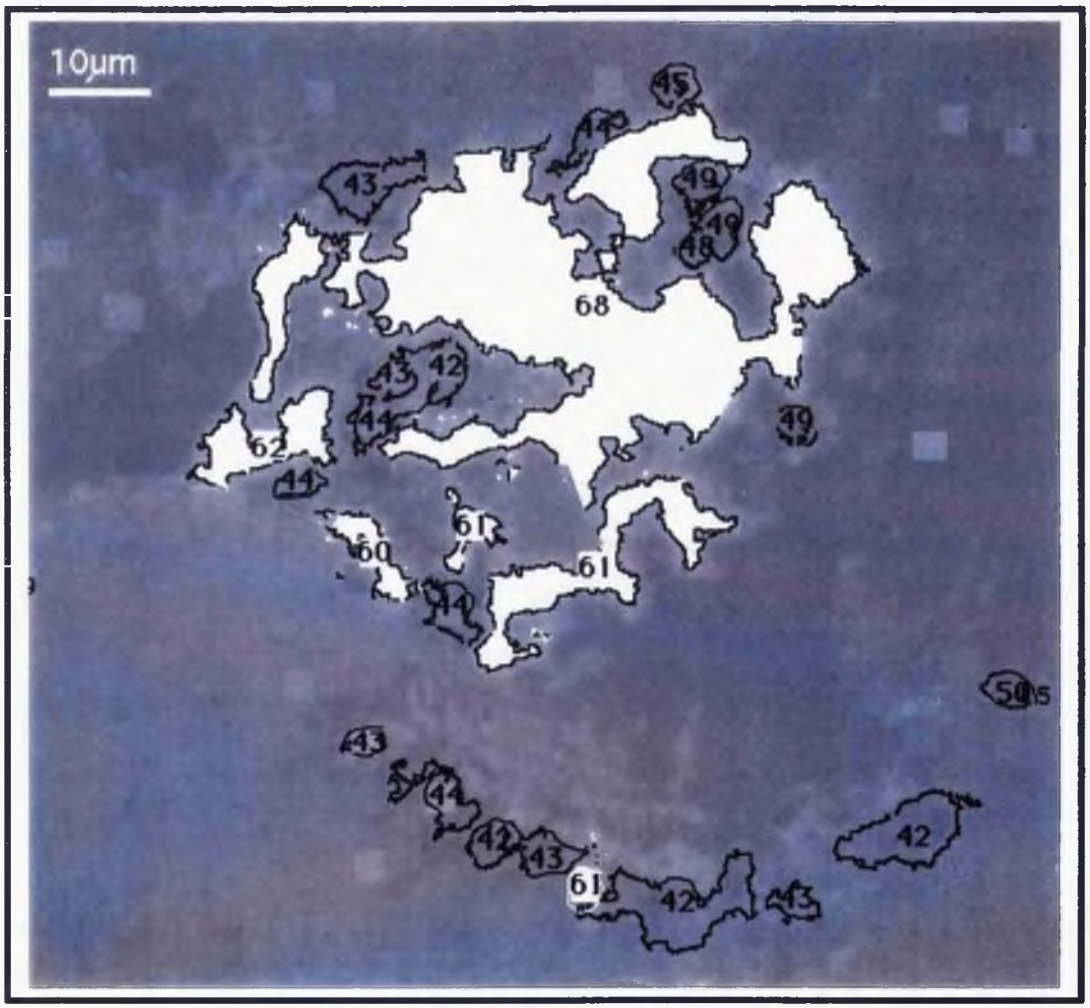


Figure 52:

Contour map (isopach) (50x-objective) for Endothelial cell B10D(2) was cultured in 4%Dextran 503,000, bars 10μm.

Cell appeared with unequal distribution of light regions and dark regions. The white colour areas in the image down indicates separation distances further away than the value in nm marked on the image, ranged > 60 - 68nm.

The dark areas indicates separation distances closer than the indicated number marked on the image in nm which were ranged between < 42 and 50nm.



II- Grooved topography

Figure 53:
IRM images (50x-Ojective) show endothelial cells B10D(2) that were grown for 24h in Dextran 503,000 on shallow topography, quartz with 280-420 nm depth and 10 μm width

1. Control
2. Dextran 2%
3. Dextran 4%

Endothelial cells appeared in high concentration of Dextran to be smaller in size with more dark regions than cells in control medium.

Scale bars 10 μm.

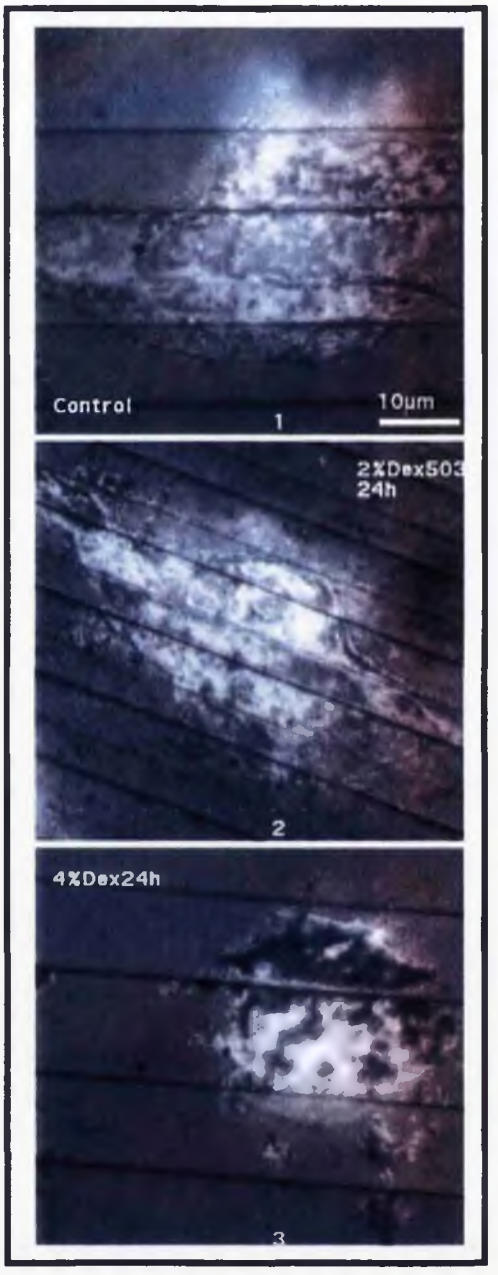


Figure 54:

IRM images (50x-Ojective) show endothelial cells B10D(2) that were grown for 3h in Dextran 503,000 on shallow topography, quartz with 280-420nm depth and 10 μm width

1. Control
2. Dextran 1%
3. Dextran 2%
4. Dextran 4%

Endothelial cells cultured in (high viscosity medium) high concentration of Dextran appeared to be smaller in size with more dark regions than cells cultured in control medium, see picture 3.

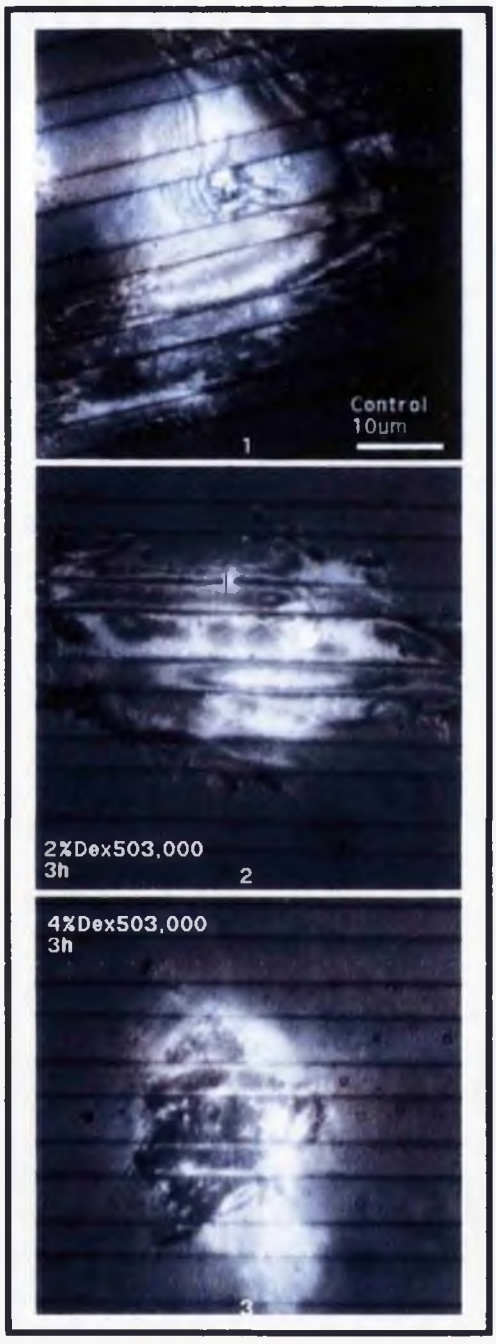


Figure 55:

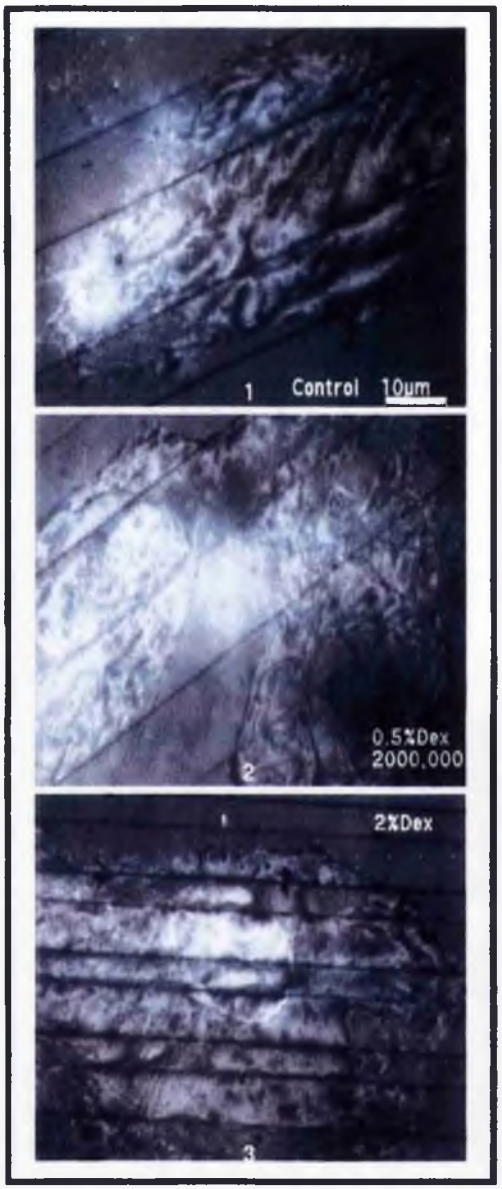
IRM images (50x-Ojective) show endothelial cells B10D(2) that were grown for 24h in Dextran 2000,000 on shallow topography, quartz with 280-420nm depth and 5 μm width

1. Control
2. Dextran 0.5%
3. Dextran 2%

Endothelial cells cultured in high concentration of Dextran appeared to be darker than cells cultured in control medium

Figure 56:

IRM images (50x-Ojective) show endothelial cells B10D(2) that were grown for 3h in Dextran 2000,000 on shallow topography, quartz with 280-420nm depth and 5-10 μm width



1. Control
2. Dextran 0.5%
3. Dextran 2%

There is not much difference between Endothelial cells cultured in Dextran and those cultured in control medium for only 3h.

Figure 57:

IRM images (50x-Ojective) show endothelial cells B10D(2) that were grown for 24h in Ficoll 400,000 on shallow topography, quartz with 280-420nm depth and 10 μm width

1. Control
2. Ficoll 2%
3. Ficoll 6%
4. Ficoll 8%

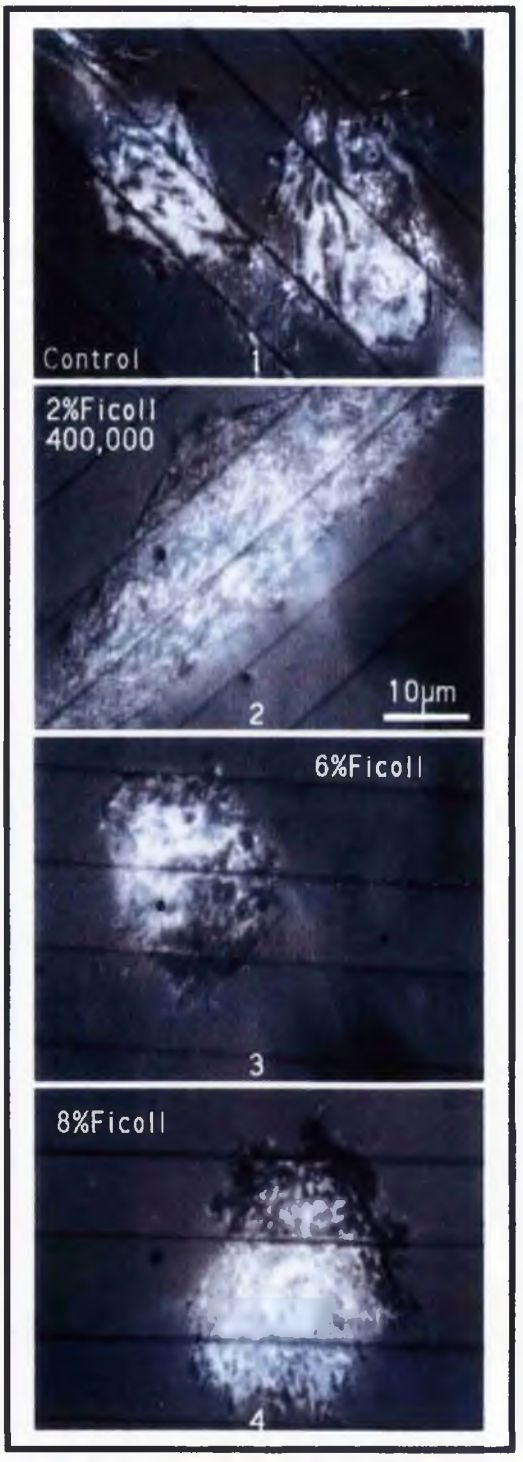
Pictures 3 and 4 show that Endothelial cells decreased in size with increasing concentration of Ficoll (of high molecular weight) in the medium.

Figure 58:

IRM images (50x-Ojective) show endothelial cells B10D(2) that were grown for 3h in Ficoll 400,000 on shallow topography, quartz with 280-420nm depth and 10 μm width

1. Control
2. Ficoll2%
3. Ficoll6%
4. Ficoll8%

Pictures 3 and 4 show that Endothelial cells decreased in size with increasing Ficoll concentration even at 3h.



5. Live IRM study

Live IRM was used as it gives advantages over fixed techniques due to continuous, artefact free, cell imaging, allowing observations of cell behaviour during adhesion to substrate in the presence of medium of high viscosity.

Results:

Cells were incubated for 24h in ordinary control media (ECT for Epitenon and Ham's F10 for Endothelial) in the modified petri dishes (see Materials and Methods).
For live IRM observations the control media were changed to Ham's F10 for both cell types, because this type of media does not require CO_2 which is difficult to add during the live experiments. Cells were observed in this media for 30-40min as a control, then the experimental media were added to the experimental dishes and observed for more than 3 hours.
The explanations in the figures legend are best demonstrated in these experimental results (Fig 59-66).
To conclude the observation, cells appear to shrink especially in high concentrations of the reagents of highly viscous media.
Live observations of cells show that cells, which were cultured in media of high viscosity, appeared to still grow, migrate and spread but at lesser rates than in control media. It has also been shown that cells do divide in medium of high viscosity, as shown in Epitenon 4% Ficoll 400,000 (the movie).
It is also observed that Epitenon cells might be more resistant to viscosity changes than Endothelial cells. They can adapt to the medium of very high viscosity (8% Ficoll 400,000), see fig 62 for epitenon and fig 64 for endothelial last picture for both. (This was clear in the related Figures and clearer if you see the submitted CD disc).

Colour maps

A computer program was used to generate pseudocolour maps for the time-lapse images allowing the differences of one cell over time to be distinguished.

Figure 59:

Live (time-lapse) IRM images (50x-Objective) and pseudocolour map, of epitenon cells that were grown in 0.5%Dextran 2000,000.

1-The first image from the Time-lapse shows observation of control experiment in the first 30 min when cell cultured in control medium Ham's F 10, indicated by dark blue colour (time 12:10) in the pseudocolour map.

2-The second image shows experimental observation for the next 30 min after adding Dextran, indicated by pale blue (time 12:50) in the pseudocolour map.

Note that cells retract and shrink after adding Dextran which was clear in the last image.

3- Last image from the live observation of the same cell, the cell have more dark regions and shrink, indicated in the pseudocolour map with black colour (time 15:40).

I-Live IRM observation for epitenon cells:

Time-lapse images

1-Control 2- 0 Time after adding Dextran 3-Last observation

Colour map

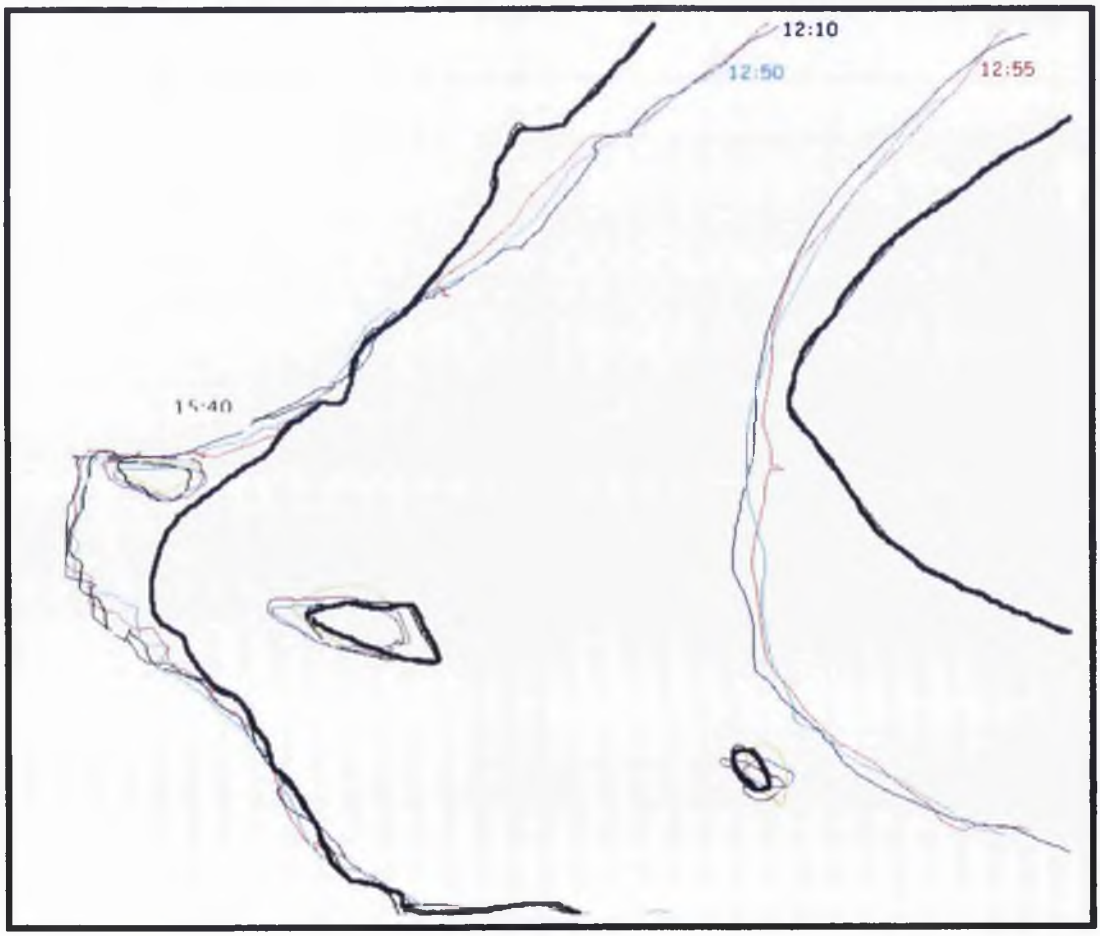


Figure 60:

Live (time-lapse) IRM images (50x-Objective) and pseudocolour map, of epitenon cells that were grown in 2% Dextran 2000,000.

The first image for the control cell that was grown in the control media (Ham's F 10) before adding Dextran, indicating with the red colour in the pseudocolour map (time 11:45).

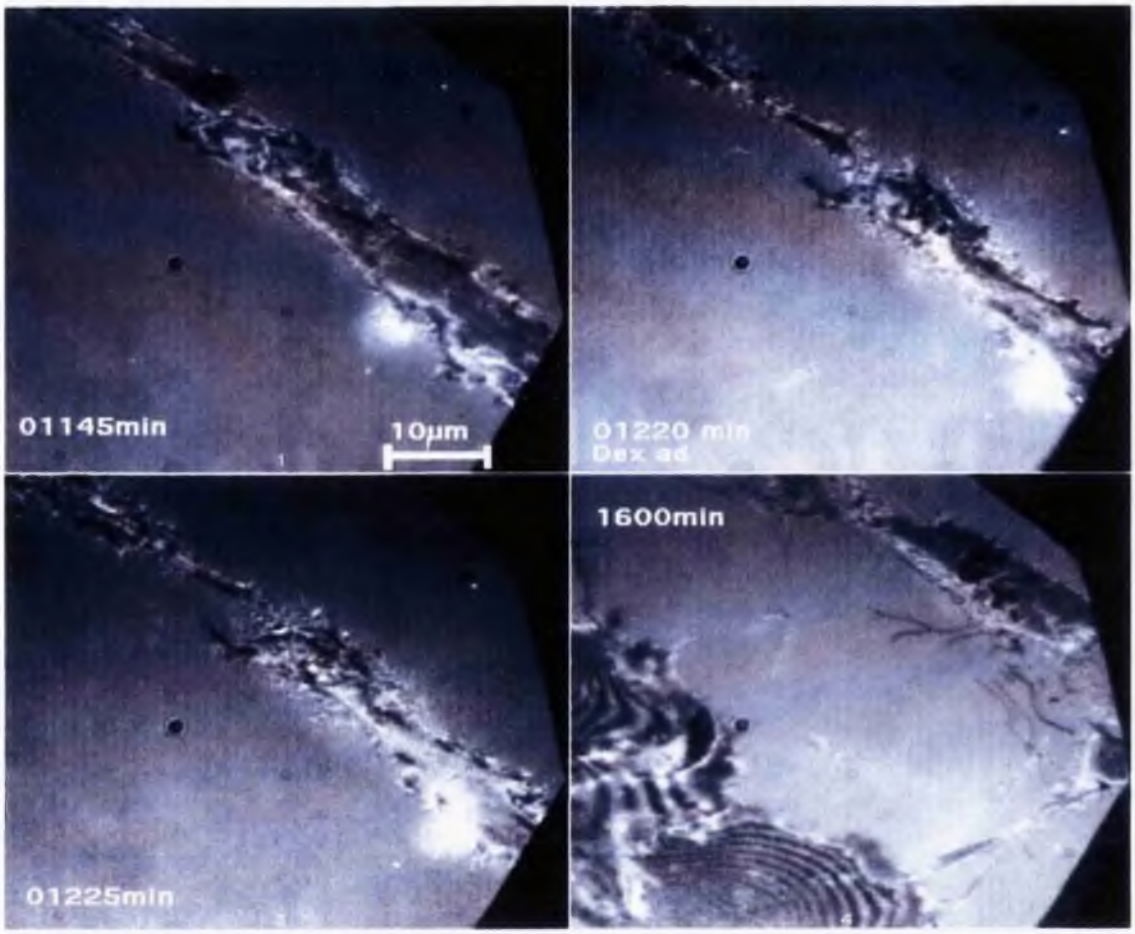
And the other images after adding Dextran, 10 min after adding Dextran indicating with the blue colour in the pseudocolour map (time 12:20 in the Time-lapse image).

After about 5 hours from the starting time of the observation cell shrink but still migrate in the field (see cell in the right top of the last picture 4) indicating by purple colour no. 5 in the pseudocolour map.

Note that the cell appeared significantly reduced in size after adding high concentration of Dextran to the medium but it still moves and migrate in the field.

In the last picture (16:00min) appear large air bubbles (down left).

Time-lapse images



Colour map

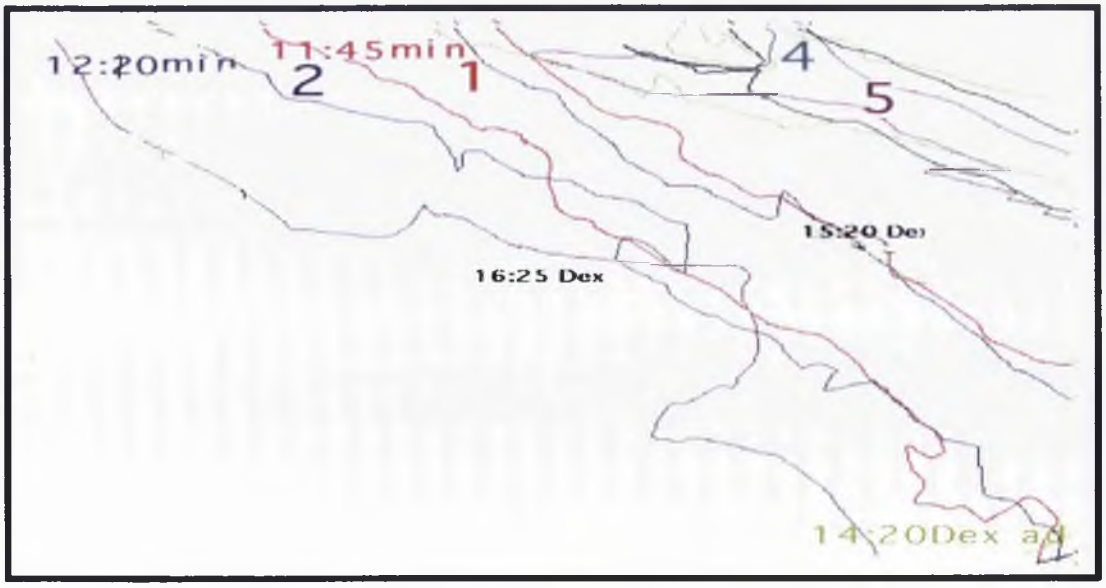


Figure 61:

Live (time-lapse) IRM images (50x-Objective) and pseudocolour map, of epitenon cells that were grown in 4% Ficoll 400,000.

The first image in 12:45 min and second image in 13:30 min for the control cell that was grown in the control media (Ham's F 10). Control cell was indicated with red colour in pseudocolour map (time 12:50 min).

The third image for the same cell after 5 min from adding Ficoll 400, 000 conc.4% which was added at 13:35.

In the last image picture 4 (time 17:15) after 3 hour and 20min from adding Ficoll cell appeared to adapted with the thick medium and started to grow again.

Note that cells still grow and spread even after adding the high viscosity medium.

It is also appear that cells can divide in medium of high viscosity as shown in Fig 58.

Time-lapse images

1-Control medium

2-Last image in control

3-Experimental 5 min after adding Ficoll

4- After more than 3hrs

Colour map

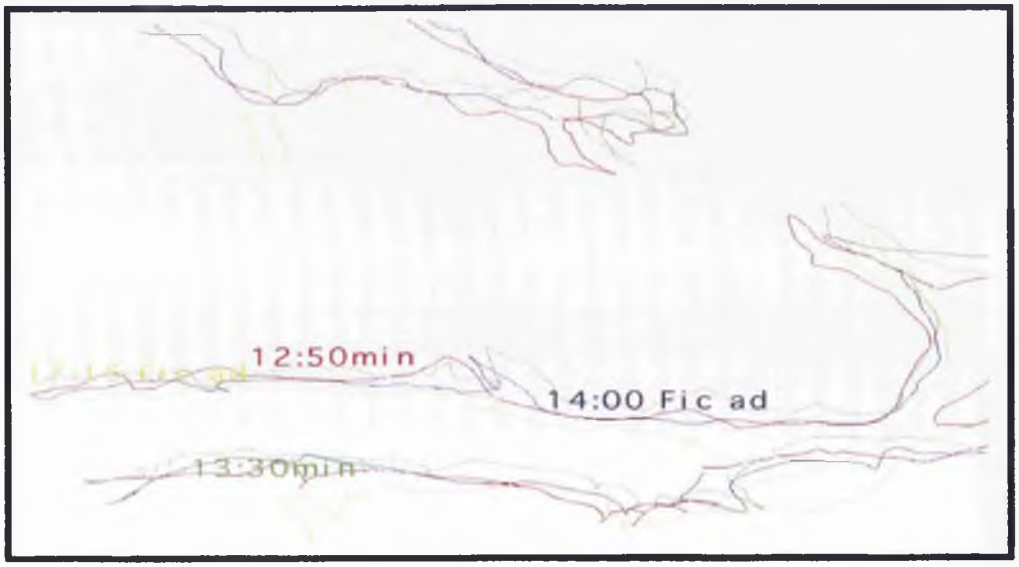
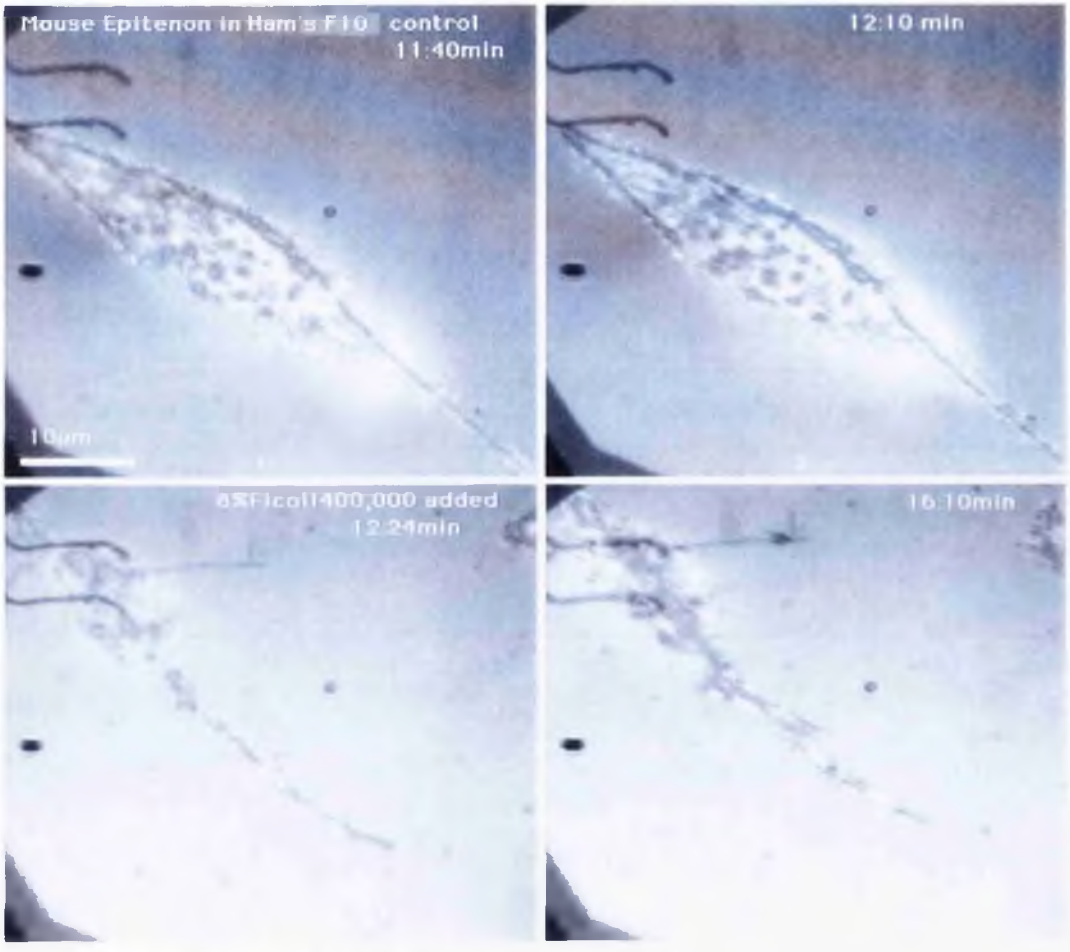


Figure 62:

Live (time-lapse) IRM images (50x-Objective) and pseudocolour map, of epitenon cells that were grown in 8 % Ficoll 400,000, which was added at 12:20min.
The first and second images for cell grown in the control media (Ham's F10) before adding Ficoll, time 11:40 min and 12:10 min of control experiment indicating with green and purple colour in the pseudocolour map.
The third image after 4 min from adding Ficoll, cell had appear to shrink fast in this medium of very high viscosity, indicated with pink and red colour in the pseudocolour map.
Note that (in the last picture of the figure) cell adapt with the viscous medium and start to grow but of course at lesser rates than in control medium. This indicates that epitenon cells may be able to live in viscous media.

Time-lapse images



Colour map

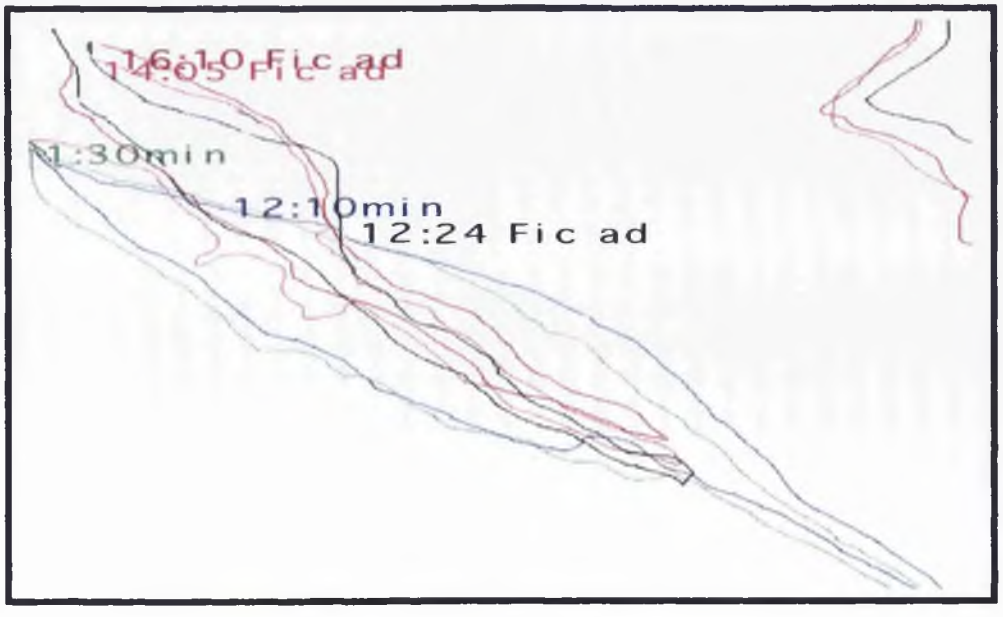


Figure 63:

Live (time-lapse) IRM images (50x-Objective) and pseudocolour map, of mouse endothelial cells B10D(2)

The first image (at zero time) for cells were grown in control media (Ham's F10) before adding Ficoll, and the second image after 30-min of observation in the control medium, indicated with 12:00 and 12:30 in the time-lapse (picture1 & 2) and with dark blue colour in the pseudocolour map.

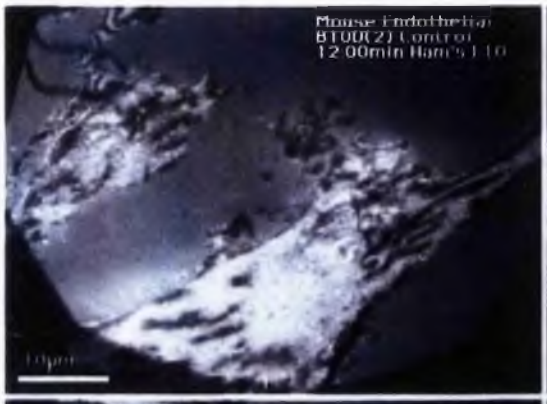
The third image after adding 4% Ficoll 400,000 which was added at 12:35 min for the experiment (picture 3 in the time-lapse), dark regions inside the cells increased in this 5 min after adding Ficoll.

The last image (picture 4) from time-lapse after about two hours from adding Ficoll, cells appear to shrink and lose some cellular processes (lamellipodia), see white arrows in this stage of the experiment, indicated with the green colour in the pseudocolour map.

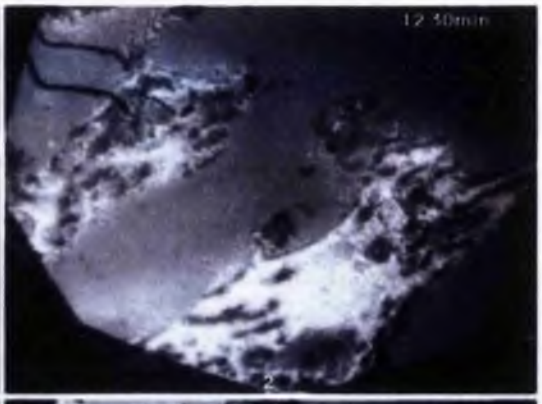
II-Live IRM observation for endothelial cells B10D(2) :

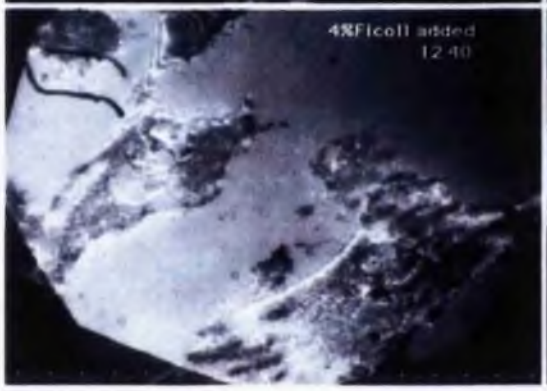
Time-lapse

0 time

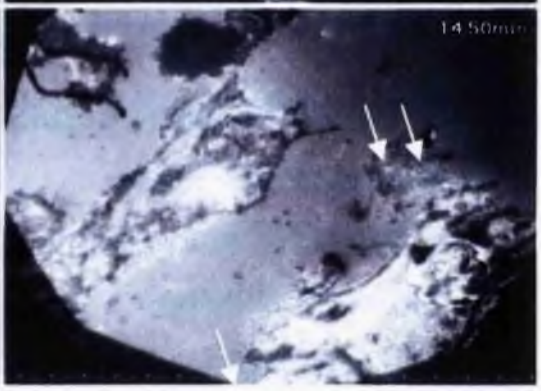


after 30min in control medium





5 min after adding Ficoll



last after 2 hrs & 10 min from adding Ficoll

Colour map

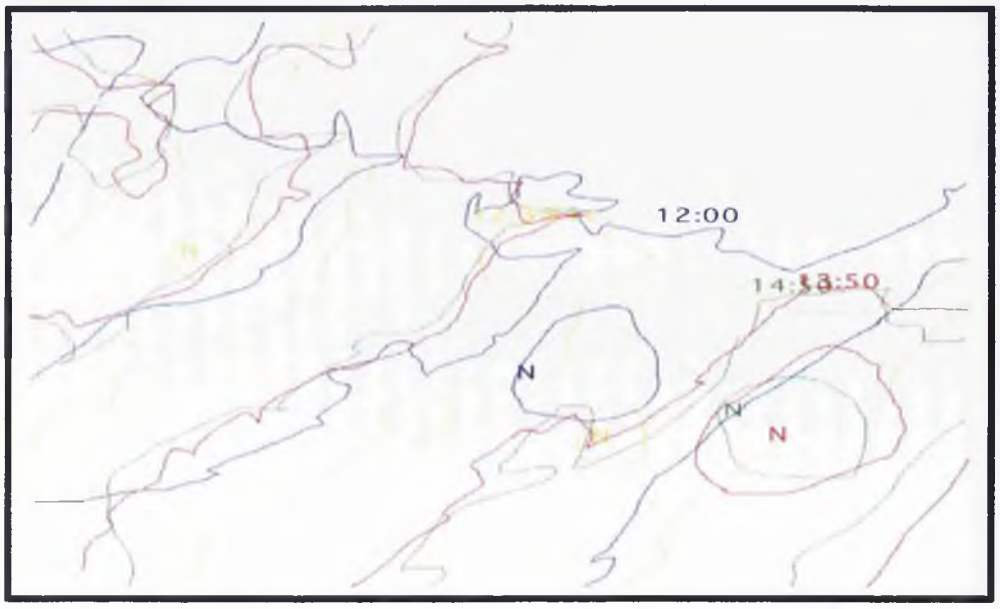


Figure 64:

Live (time-lapse) IRM images (50x-Objective) and pseudocolour map, of mouse endothelial cells B10D(2)

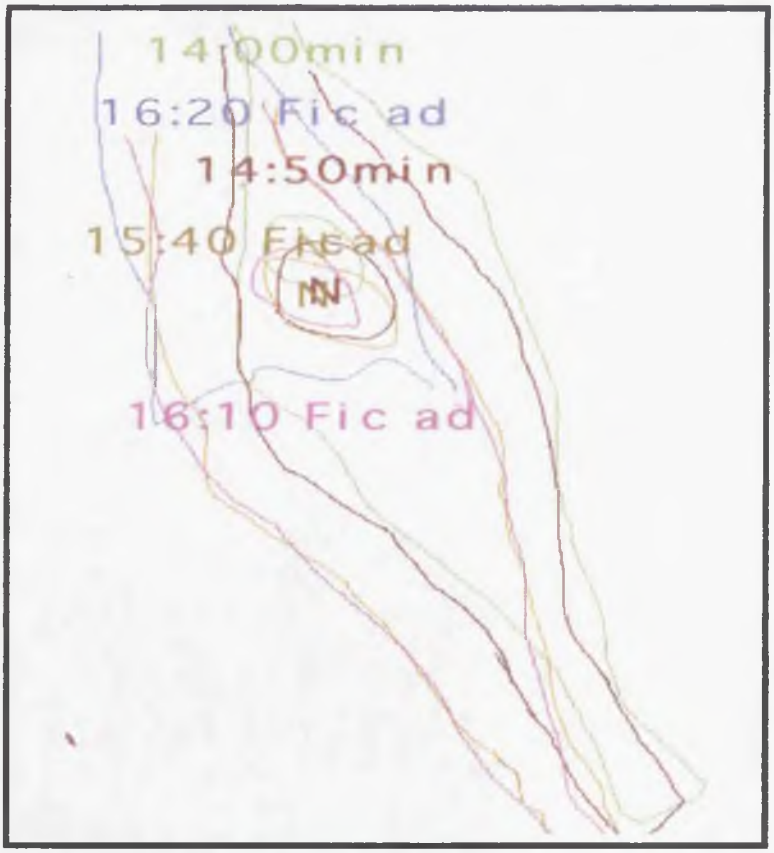
The first image for cell was grown in control medium (Ham's F10) before adding 8%Ficoll 400,000. Indicated with the time 13:50 (picture 1) in the time-lapse images and with green colour in the pseudocolour map.

The second image (picture 2) at 14:55 exactly after adding Ficoll, which was added at 14:54min.

The third image (picture 3) after about 15 min from adding Ficoll, indicated by the pink colour in the pseudocolour map, cell start to shrink at this image.

The last image (picture 4) after one hour and 25 min from adding Ficoll, indicating by the brown colour in the pseudocolour map, cell rupture in this stage may by due to the effect of very highly viscous medium on it or related to osmotic shock.

Colour map



Time-lapse

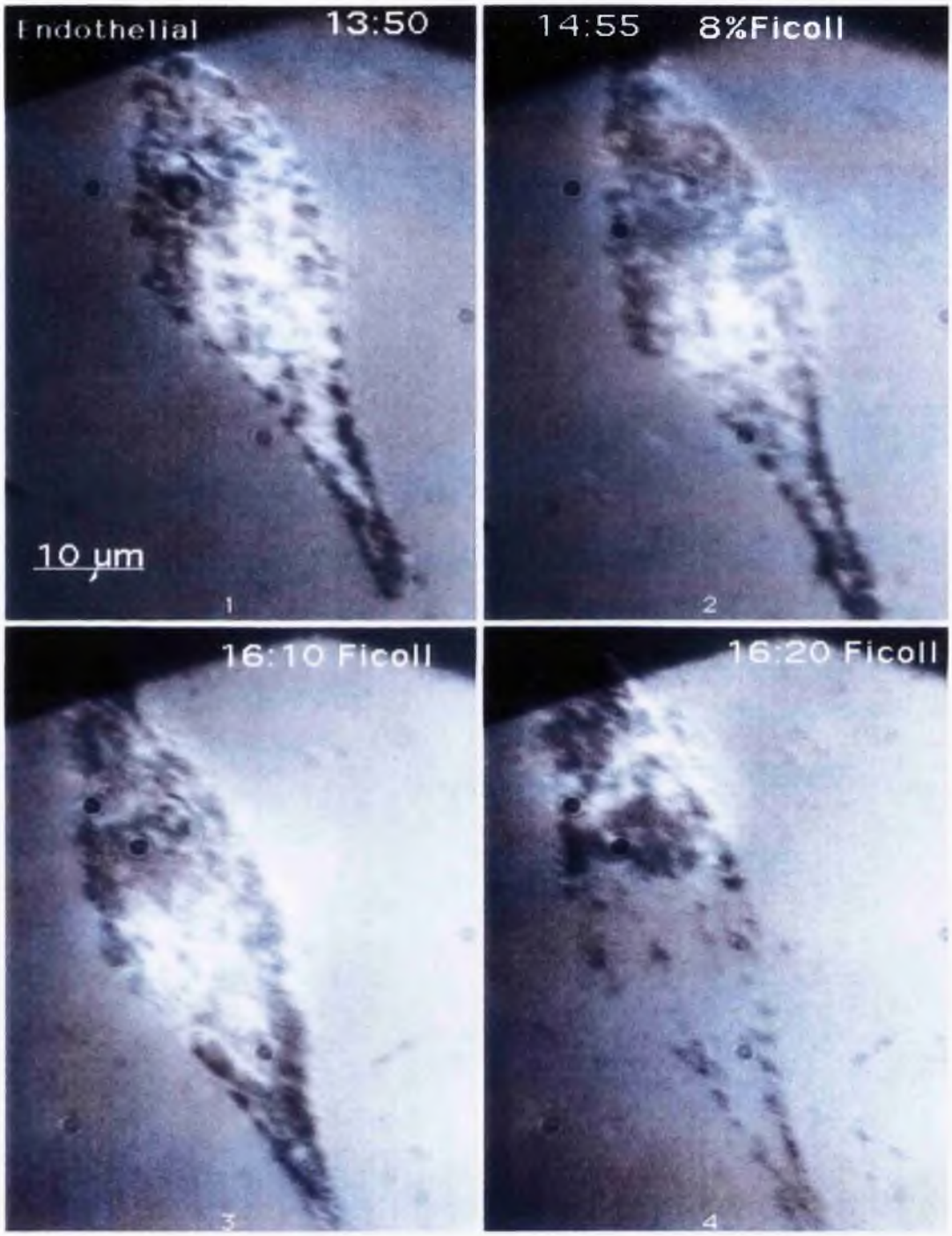
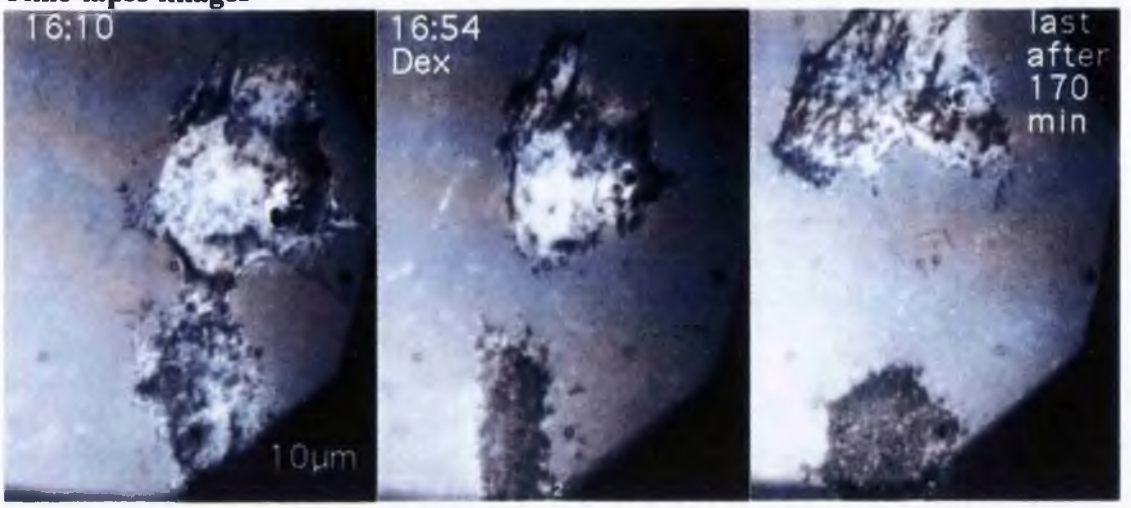

Endothelial
13:50
10 µm
1
14:55
8%Ficoll
2
16:10 Ficoll
3
16:20 Ficoll
4

Figure 65:
Live (time-lapse) IRM images (50x-Objective) and pseudocolour map, of mouse endothelial cells B10D(2).
The first image (picture 1) for cells that were grown in the control medium (Ham's F 10) before adding Dextran, indicated by the red colour in the pseudocolour map.
The second image after adding 0.5% Dextran 2000,000, added at 16:54min.
The last image (picture 3) after 170min, indicating with the blue colour in the pseudocolour map at 17:40 min from start of the experiment.
The cells appear to shrink and became dark in the Dextran medium.

Time-lapse images



Colour map

Figure 66:

Live (time-lapse) IRM images (50x-Objective) and pseudocolour map, of mouse endothelial cells B10D(2).

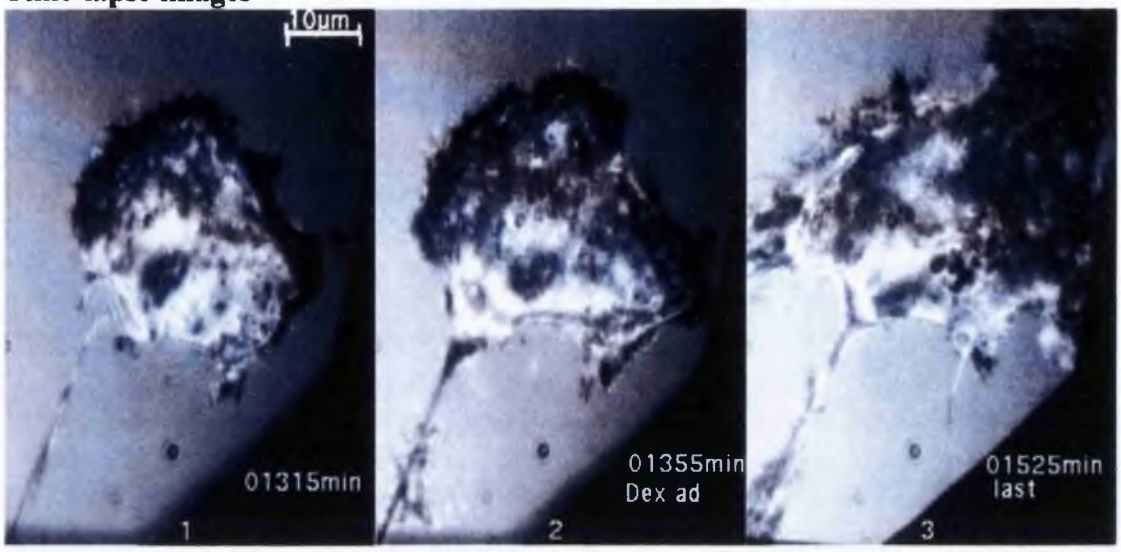
The first image (picture 1) for cells that were grown in the control medium (Ham's F 10) before adding Dextran, indicated by the yellow colour in the pseudocolour map.

The second image (picture2) after adding 2 % Dextran 2000,000, added at 13:55min, indicating by red colour in the pseudocolour map.

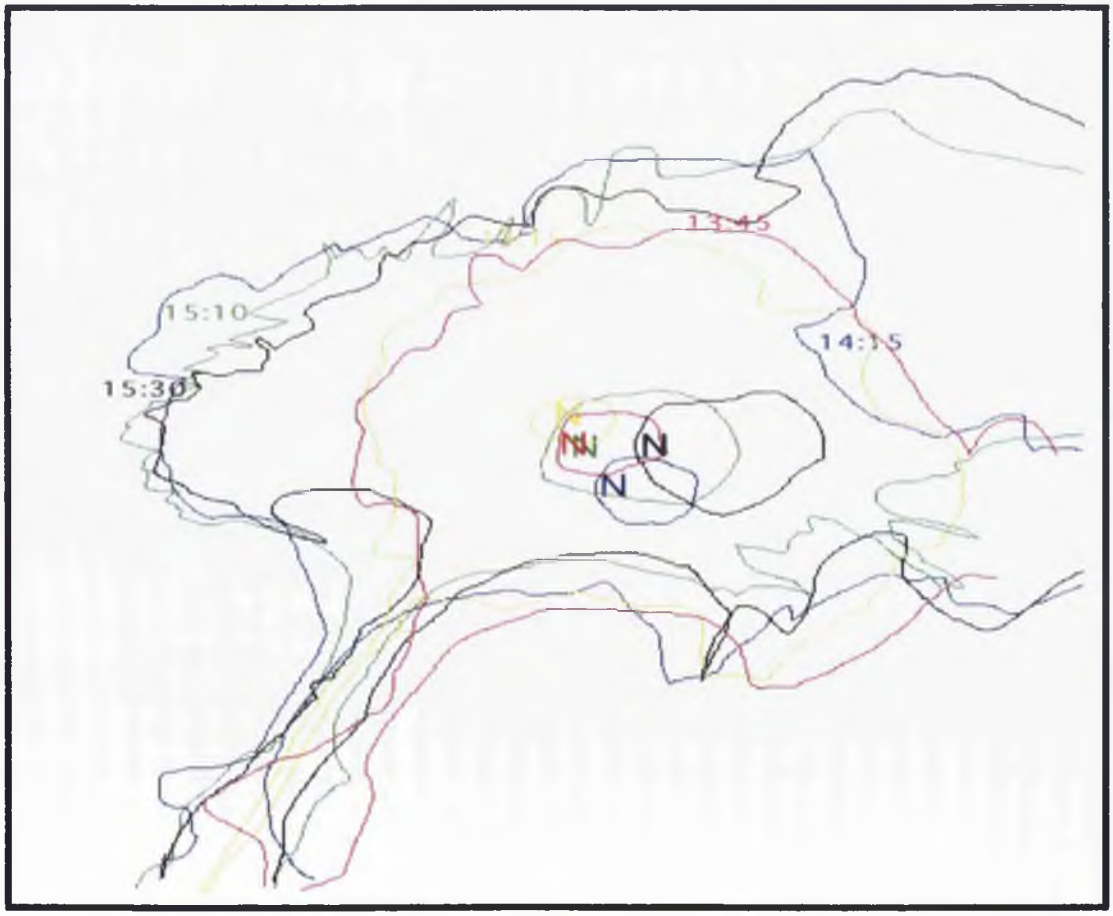
The last image (picture 3) in the time 15:25, after one and half hour from adding Dextran indicated with blue colour in the pseudocolour map.

Cells still are apparently alive in the viscous medium and have many processes as seen in the last picture.

Time-lapse images



Colour map



Discussion and conclusion

It has been shown from the IRM study that cells had more dark regions when the viscosity of the medium increased. In very highly viscous media cells became less in number, very small in size with many first order (black and white) fringes around them. To investigate this more, isopach were calculated from the measured refractive index of high concentration of given reagents. They show that the cells have close approaches of 34 nm to the surface and furthermore that growth in high viscosity medium does change this to closer contacts.

Growing the cells on groove/ridge topography normally allows a considerable cellular reaction to the topography but this is inhibited by medium of high viscosity. Several explanations of this can be proposed. Chief amongst these are

1. The concept that cells are not energetic enough to move against the high viscosity of the medium.
2. The concept that diffusion processes, especially under the cells are altered by the high viscosity medium perhaps changing cell metabolism so the crawling activities are damaged.

The first is unlikely to be true because the cells might move more slowly spreading should eventually take place, see live IRM results.

The second suggests several tests: Increasing the viscosity should reduce reaction to topography, and Stirring the medium or agitating it might enhance reaction to topography.

But are cells at the same distance from the substratum in the presence of high viscosity media as in low viscosity media?

Present results with IRM suggest that the cells on flat surfaces show change in separation from the glass surface when the medium viscosity is changed. This is that the cells retracted slightly when the high viscosity medium was added. This may lead to the appearance of fringes, which represent the 'overhang' of the cells over the surface. The dark parts of the image became darker but this is probably consistent with the changes in refractive index of the medium when the viscosity materials are added. However, this needs careful checking by more experiments.

6. CELL MOVEMENT IN HIGHLY VISCOUS MEDIA

Aims

The possibility that cells can move in medium of high viscosity has been explored experimentally. Cell motility was analysed using time-lapse video phase-contrast microscopy and an Excel program (see Materials and Methods).

Results

Cells appeared to move and migrate in the highly viscous media, but the rate of movement differs according to the concentration of high molecular weight reagents in the media.

In the low concentration of reagent cells appeared to move and migrate at the same rate as a control while in the high concentration of reagent the movement rate decreased with increasing concentration of the reagent in the medium. (See the related Table 14 - 18 and Figures 67 -81).

It can be concluded from the results (in the Tables) that effects on endothelial cells are more pronounced than on epitenon cells, see for example Table 14 and Table 16. See also related movement analysis (Excel files) in the submitted CD disc for more details.

Table 14:

The Table shows computer analysis (Excel program- spread sheet developed by Dr. Mathis Riehle) for the movement experiment of Endothelial B10D(2) cells cultured in control Ham's F10 and 2%Dextran 2000,000 and video recorded for 24h (P-value calculated separately according to Mould, 1998)

Comparisons \ Cells & Media name		Endothelial, control Ham's F10	2%Dextran2000,000
Distance(μm)	Average	274.7	137.6
	SD	274	80
	P-value	P < 0.10	
Persistence	Average	0.46	0.47
	SD	0.27	0.20
	P-value	P < 0.90	
Avg velocity [μm/h] (overall)	Average	28.9	14.5
	SD	29	8
	P-value	P < 0.10	
Average velocity-0 Without stops.	Average	68.9	38.0
	SD	70	13
	P-value	P < 0.10	
Avg step (size in microns)	Average	6.2	6.3
	SD	3	2
	P-value	P < 0.90	

Avg step (size in microns): movement of cells from frame to frame.

N = 20 cells as experimental and 20 cells as control.

Table 15:

The Table shows computer analysis (Excel program) for the movement experiment of Endothelial B10D(2) cells cultured in control Ham's F10 and 4%Dextran 503,000 and video recorded for 24h (P-value calculated separately according to Mould, 1998)

Cells & Media name / Comparisons		Endothelial, control Ham's F10	4%Dextran 503,000
Distance(μm)	Average	432.6	74.8
	SD	35	34
	P-value	Lies between 0.01 and 0.001	
Persistence	Average	0.83	0.54
	SD	0.07	0.34
	P-value	$P < 0.001$	
Avg velocity [μm/h] (overall)	Average	45.5	7.9
	SD	4	4
	P-value	Lies between 0.01 and 0.001	
Average velocity-0 Without stops.	Average	102.1	46.0
	SD	21	18
	P-value	Lies between 0.01 and 0.001	
Avg step (size in microns)	Average	15.8	8.1
	SD	3	3
	P-value	Lies between 0.01 and 0.001	

Table 16:

The Table shows computer analysis (Excel program) for the movement experiment of Endothelial B10D(2) cells cultured in control Ham's F10 and 6% Ficoll 400,000 and video recorded for 24h (P-value calculated separately according to Mould, 1998)

Comparisons \ Cells & Media name		Endothelial, control Ham's F10	6%Ficoll 400,000
Distance(μm)	Average	123.8	44.2
	SD	78	16
	P-value	Lies between 0.05 and 0.01	
Persistence	Average	0.63	0.26
	SD	0.48	0.06
	P-value	Lies between 0.05 and 0.02	
Avg velocity [μm/h] (overall)	Average	13.8	4.7
	SD	9	2
	P-value	Lies between 0.05 and 0.01	
Average velocity-0 Without stops.	Average	32.5	18.8
	SD	17	6
	P-value	Lies between 0.05 and 0.02	
Avg step (size in microns)	Average	6.1	3.1
	SD	3	1
	P-value	Lies between 0.02 and 0.01	

Table 17:

The Table shows computer analysis (Excel program) for the movement experiment of Epitenon cells cultured in control ECT and 4 % Dextran 503,000 and video recorded for 24h (P-value calculated separately according to Mould, 1998)

Comparisons \ Cells & Media name		Epitenon, control ECT	4%Dextran 503,000
Distance(μm)	Average	112.8	112.9
	SD	106	63
	P-value	P < 0.90	
Persistence	Average	0.38	0.30
	SD	0.29	0.18
	P-value	P < 0.50	
Avg velocity [μm/h] (overall)	Average	11.9	11.9
	SD	11	7
	P-value	P < 0.90	
Average velocity-0 Without stops.	Average	35.2	34.3
	SD	15	7
	P-value	P < 0.90	
Avg step (size in microns)	Average	5.7	5.8
	SD	2	1
	P-value	P < 0.90	

Table 18:

The Table shows computer analysis (Excel program) for the movement experiment of Epitenon cells cultured in control ECT and 8% Ficoll400,000 and video recorded for 24h (P-value calculated separately according to Mould, 1998)

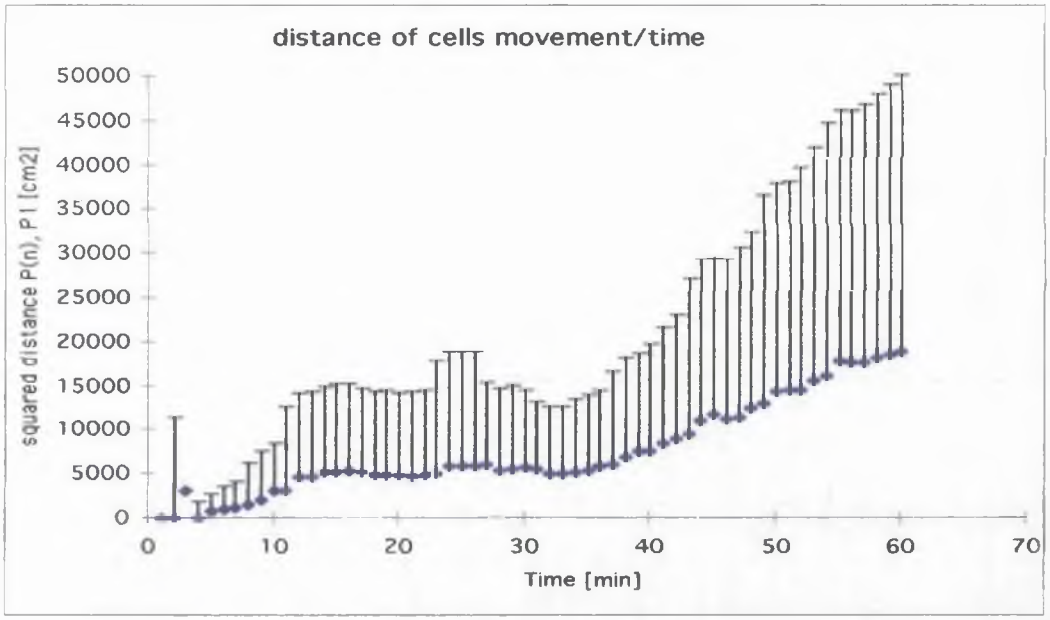
Cells & Media name / Comparisons		Epitenon, control ECT	8%Ficoll 400,000
Distance(μm)	Average	259.2	218.9
	SD	114	150
	P-value	Lies between 0.50 and 0.20	
Persistence	Average	0.30	0.40
	SD	0.25	0.38
	P-value	$P < 0.20$	
Avg velocity [μm/h] (overall)	Average	27.3	23.0
	SD	12	16
	P-value	Lies between 0.50 and 0.20	
Average velocity-0 Without stops	Average	57.7	73.4
	SD	13	34
	P-value	$P < 0.01$	
Avg step (size in microns)	Average	10.0	12.3
	SD	2	6
	P-value	Lies between 0.02 and 0.01	

6. 1. Endothelial cells B10D(2) in 2%Dextran 2000,000

Figure 67:

A large difference between control and experimental curves was found. This difference indicates that the cell move in 2%Dextran 2000,000 at clearly reduced rates than in the control medium in the same period of time.

a, control (P(n): 20 cells, P1(cm^2): indicates squared area).



b, experimental

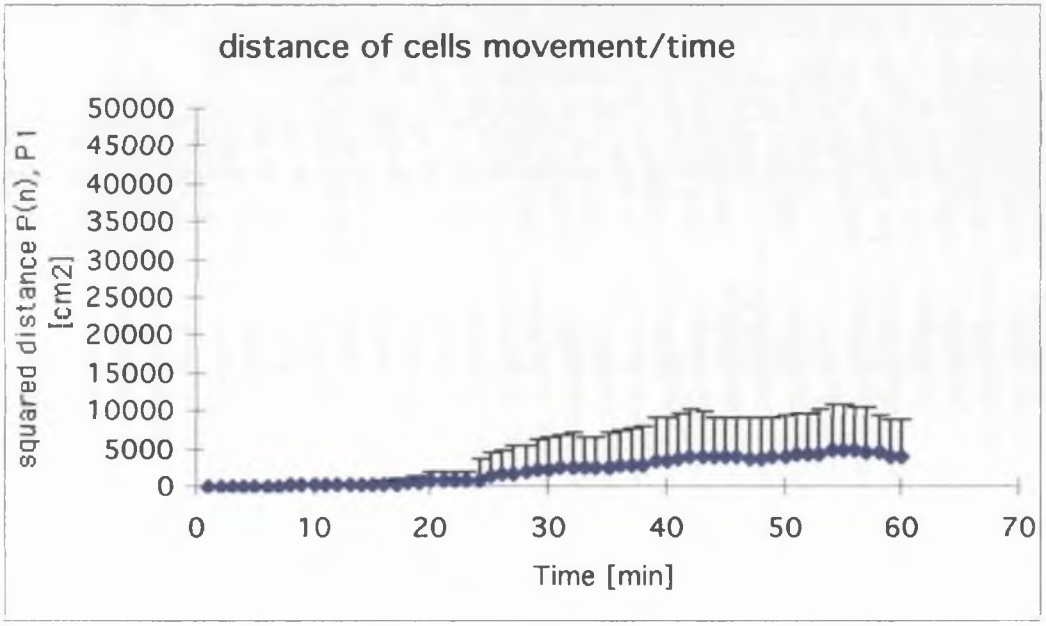
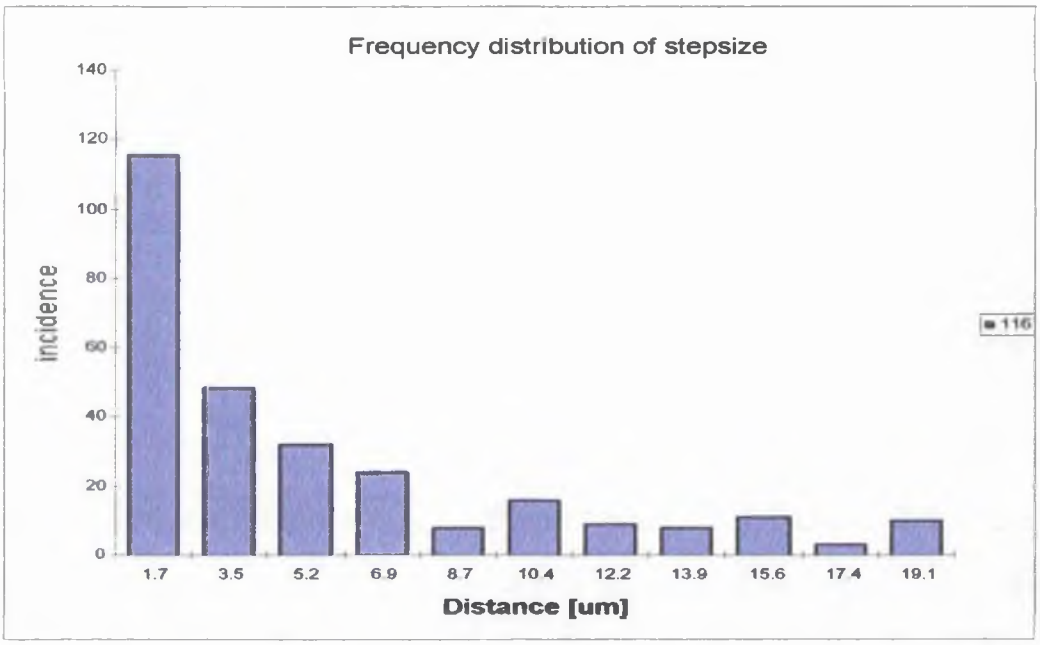
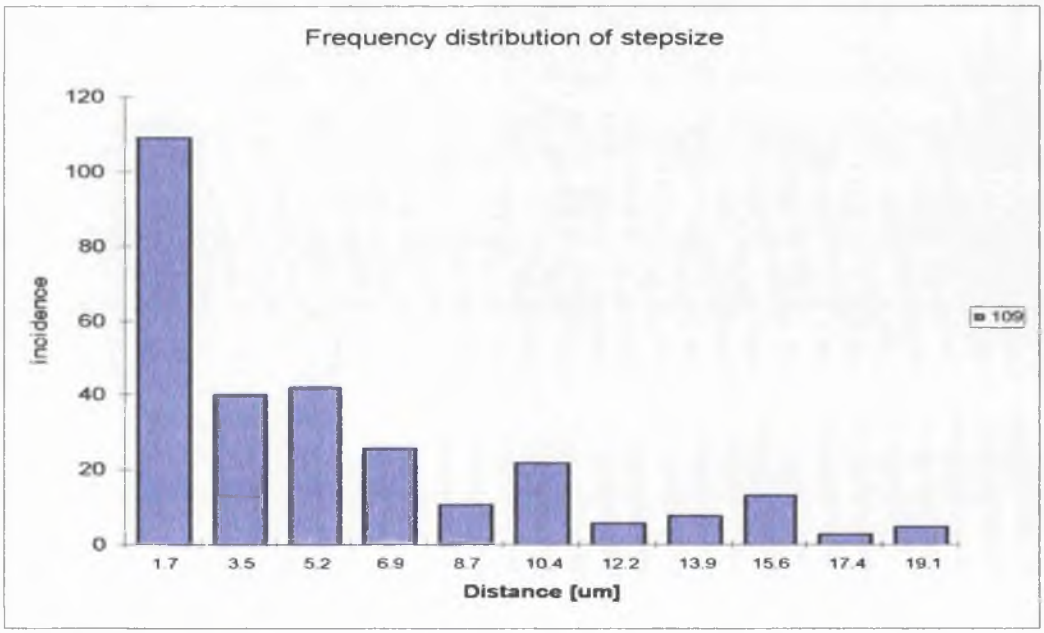


Figure 68:

The average step size of the cell cultured in 2%Dextran 2000,000 did not change from that of the Endothelial cells cultured in control medium as shown in the two graphs below.



control

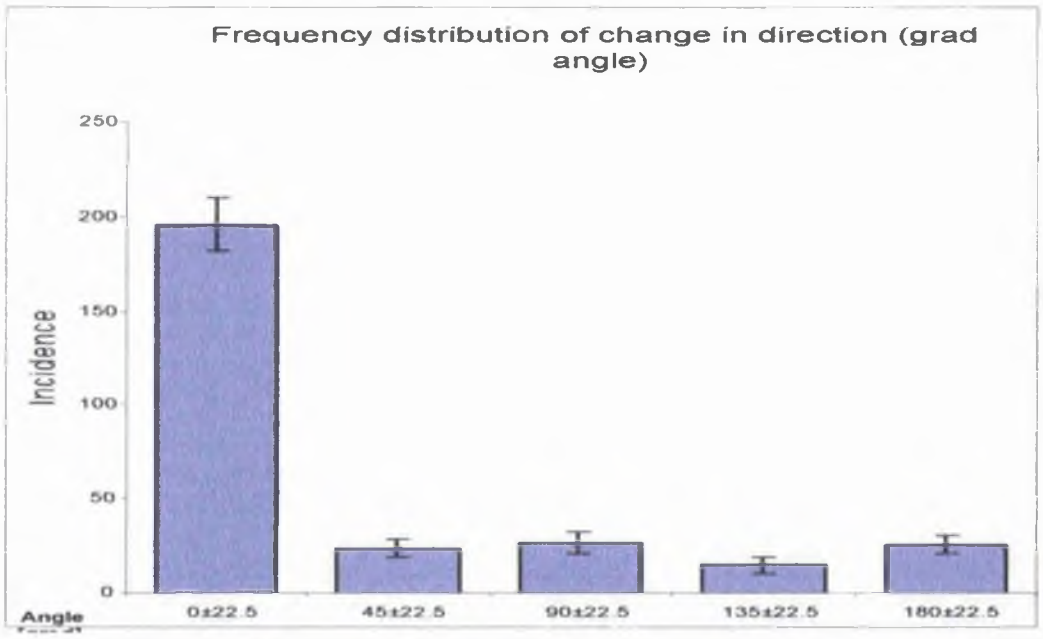


Experimental

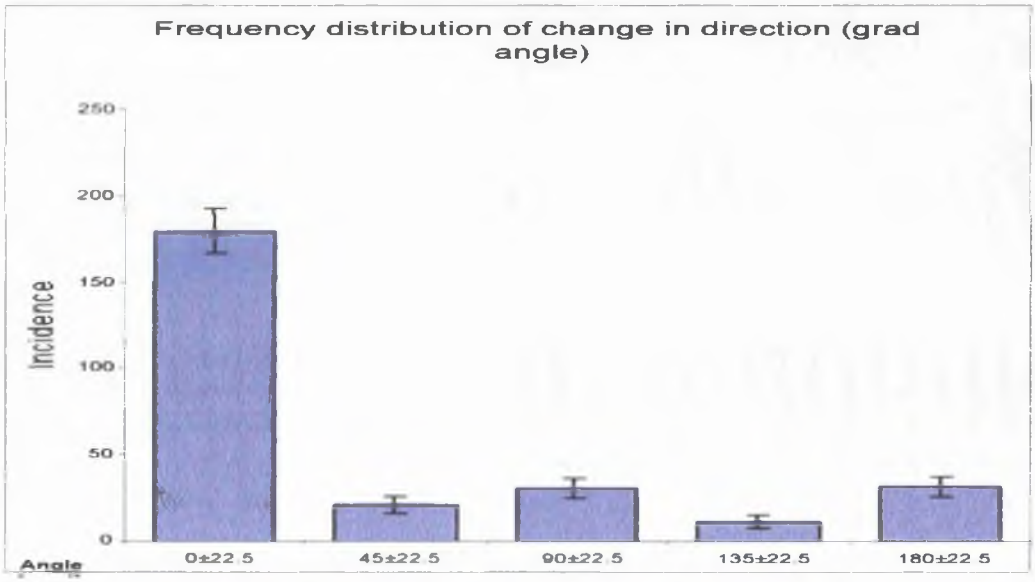
Figure 69:

The behaviour of the cells cultured in 2%Dextran 2000,000 was slightly changed from that of the Endothelial cells cultured in control medium as shown in the two graphs below.

a, control



b, experimental

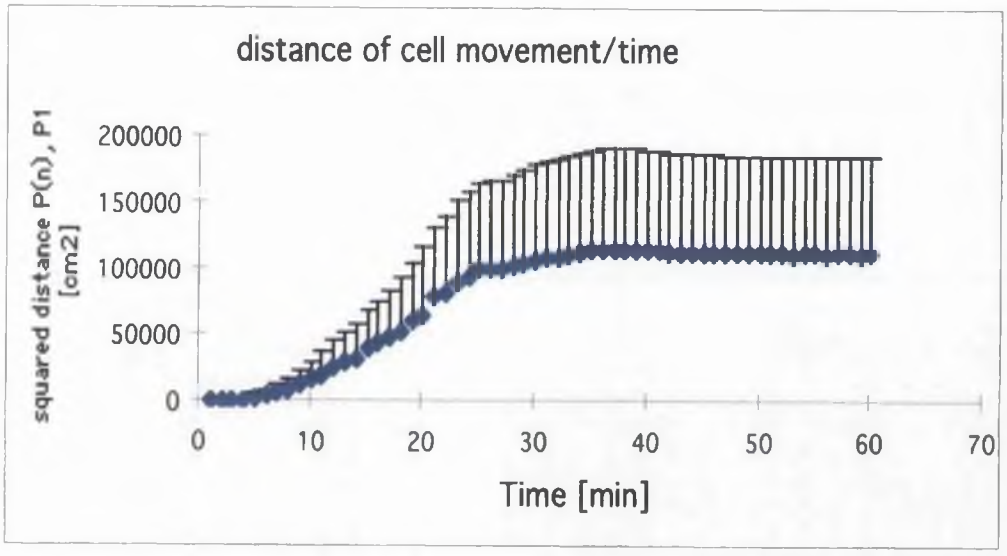


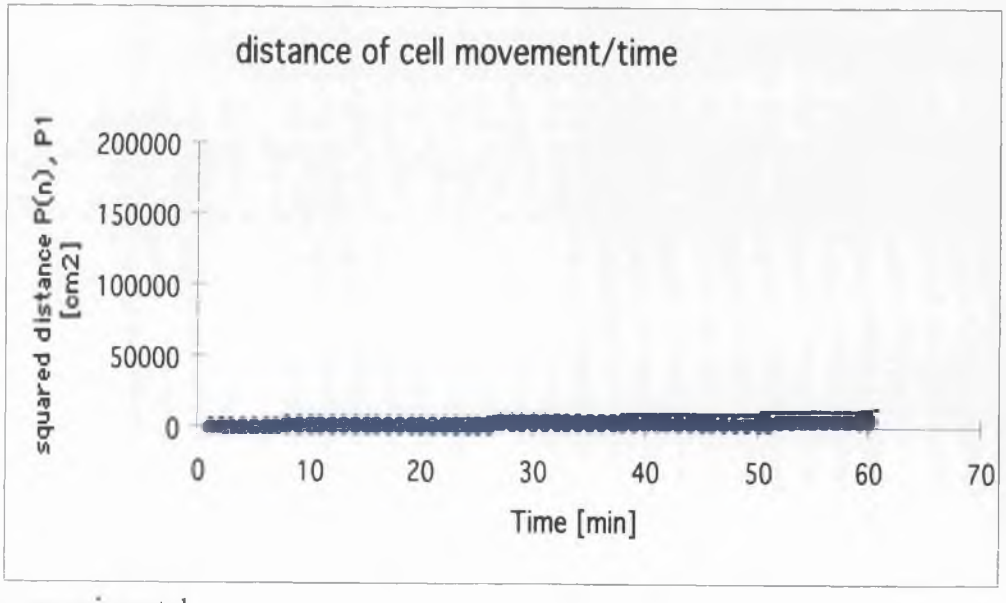
6. 2. Endothelial cells B10D(2) in 4%Dextran 503,000

Figure 70:

There is a significant difference between cells cultured in control medium (Ham's F10) and the endothelial cells which were grown in high concentration of Dextran. While cells in Dextran still move, the movement curve has sharp drop comparing with the movement curve of those cells were grown in control medium. This indicates very slight movement of the cells in 4%Dextran.

a, control

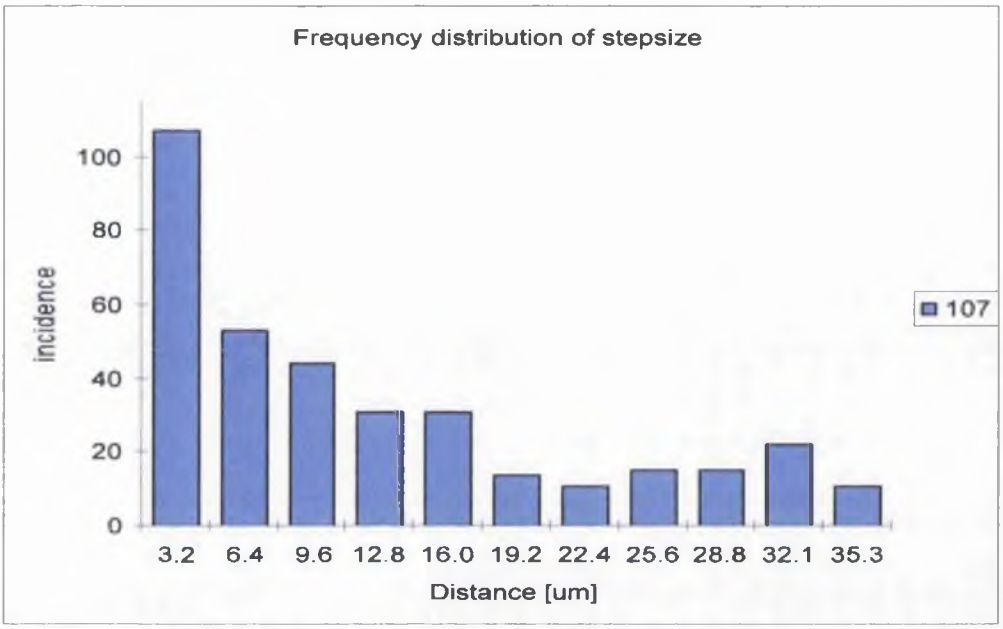




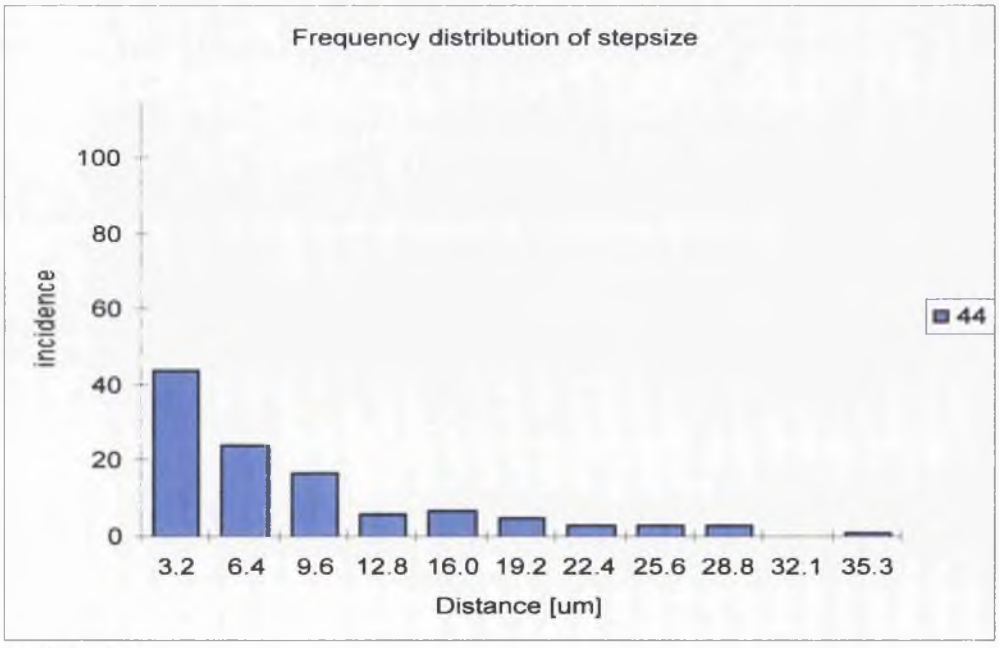
b, experimental

Figure 71:

The average step size (in micron) curve shows significant change between Endothelial cells that were cultured in control medium, first graph and those cultured in 4%Dextran 503,000, second graph.



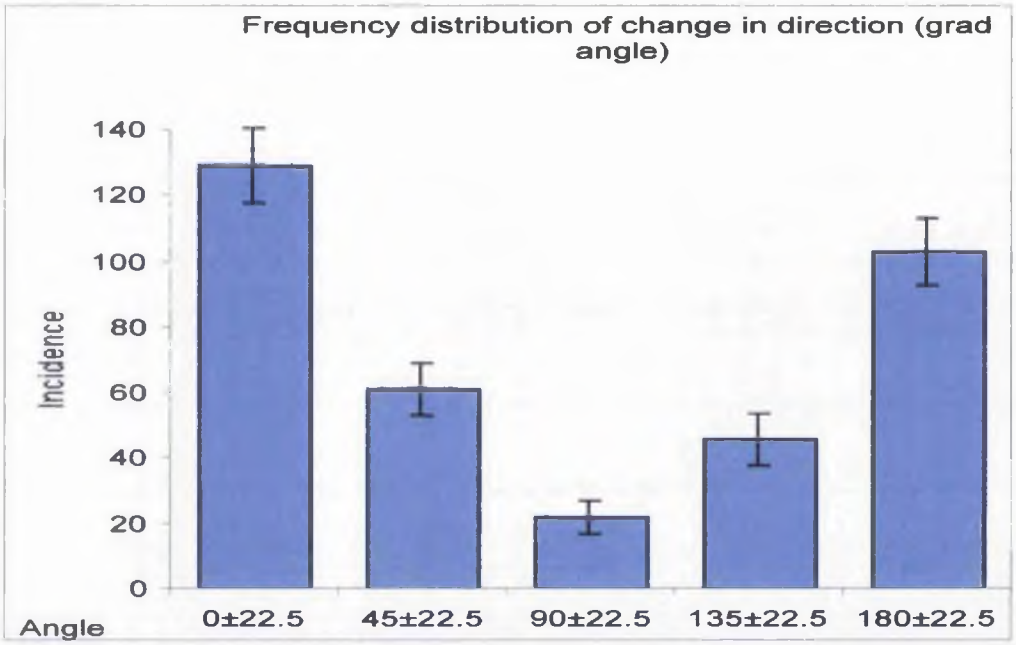
control



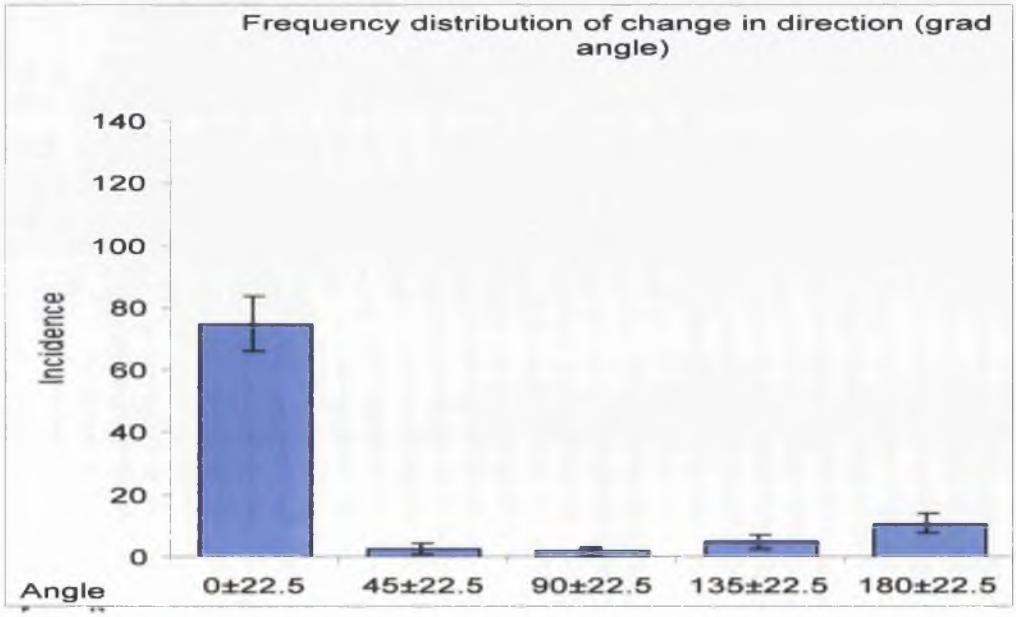
experimental

Figure 72:

Cells hardly move in 4 % Dextran 503,000, so the effect on orientation of endothelial cells B10D2 are not certain as illustrated by the graphs below.



control

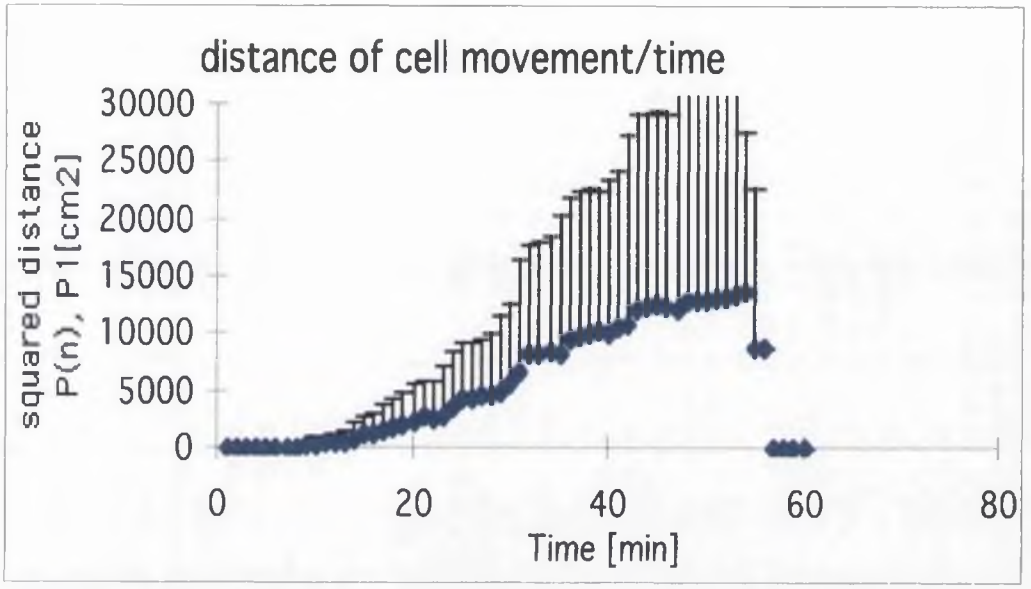


experimental.

6. 3. Endothelial cells B10D(2) in 6%Ficoll 400,000

Figure 73:

The cells cultured in medium with high viscosity (6%Ficoll400,000) show little or no movement comparing with cells cultured in control medium.



a, control
b, experimental

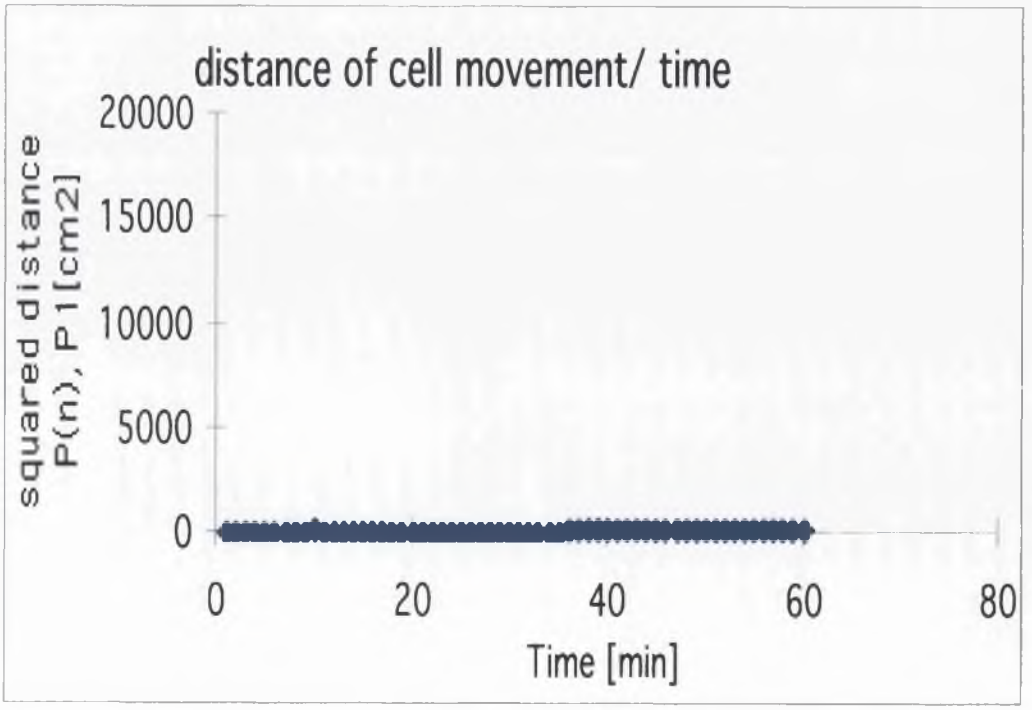
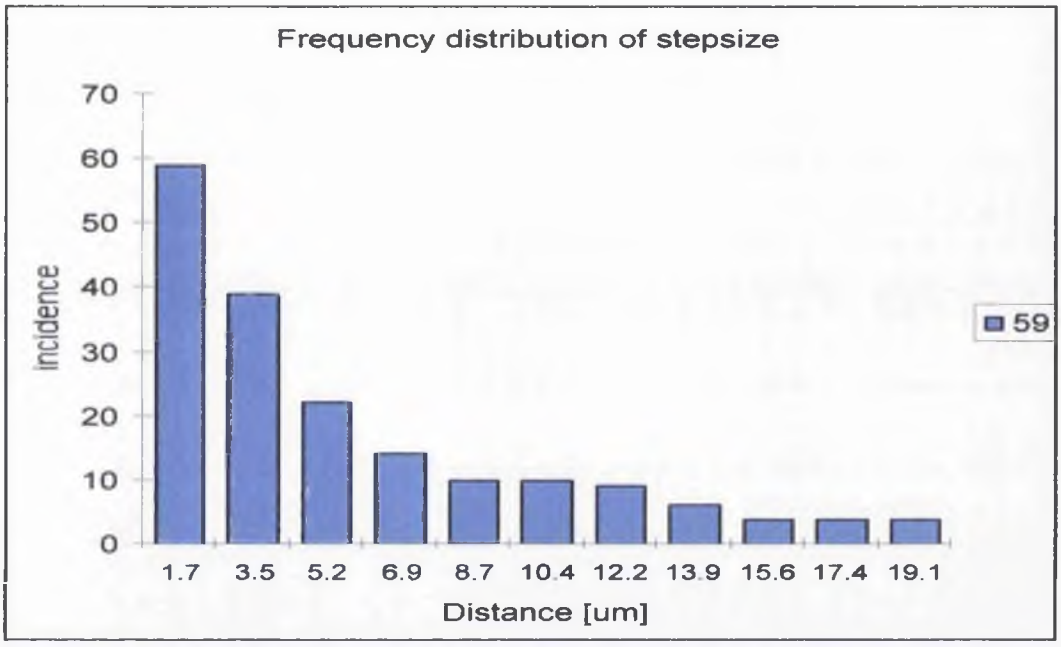


Figure 74:

It is of interest to notice that cells were cultured in medium of high viscosity (6%Ficoll400,000) shows high average of step size at the beginning of the experiment after adding Ficoll (15min) which after while became very low comparing with control cells.



control

experimental

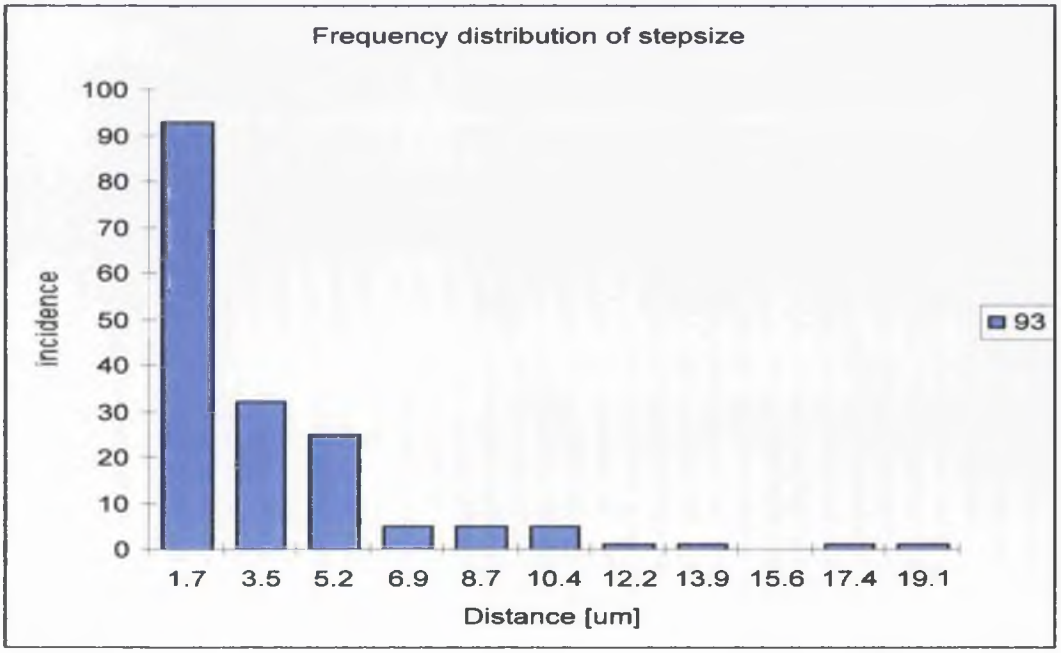
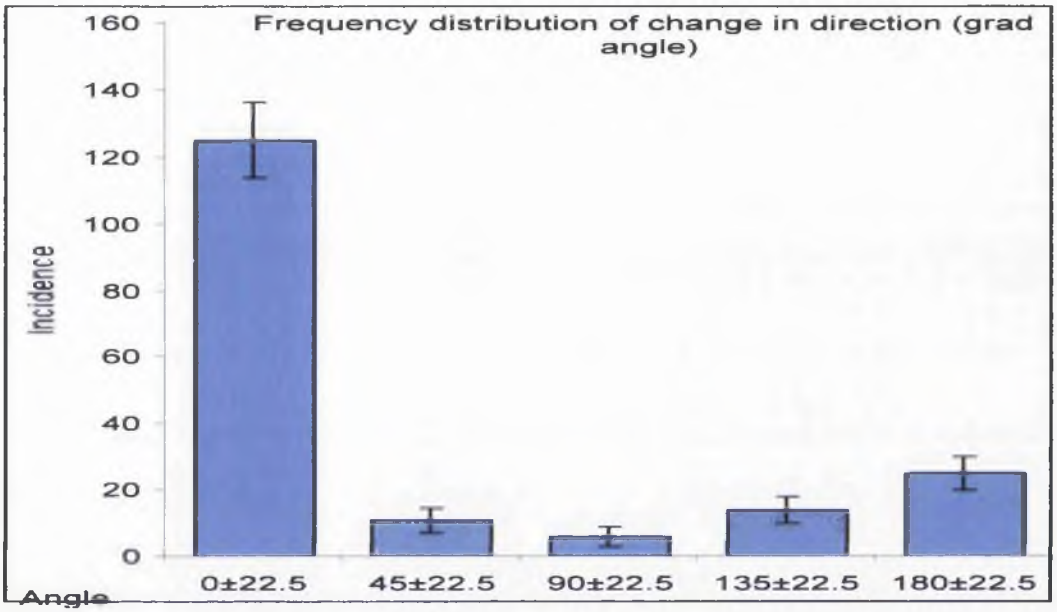


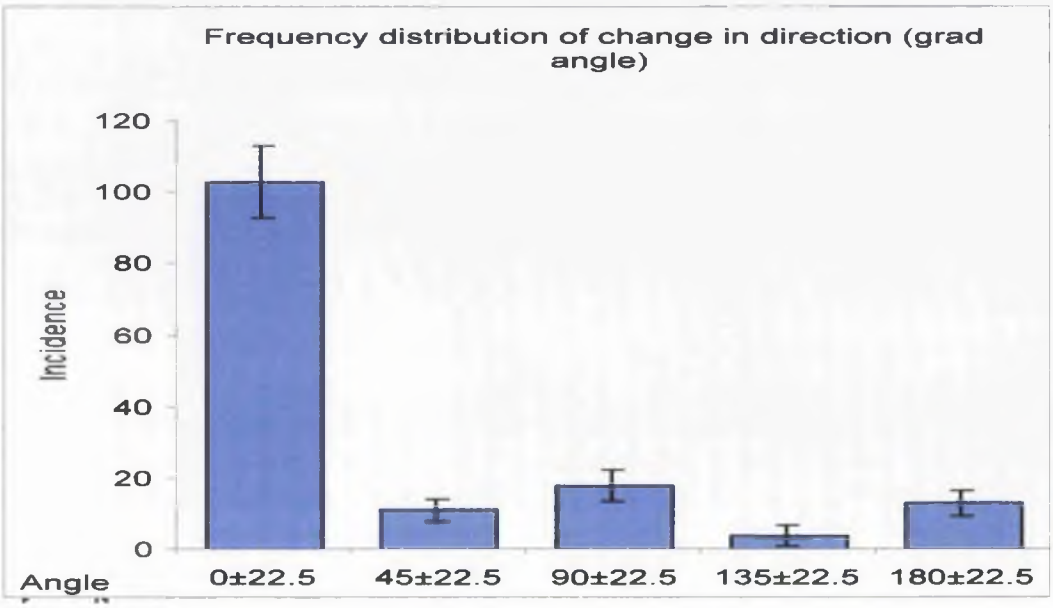
Figure 75:

There is a slight difference in the behaviour of endothelial cells when they were cultured in medium of high viscosity (6%Ficoll 400,000) comparing with those cultured in control medium.



control

experimental

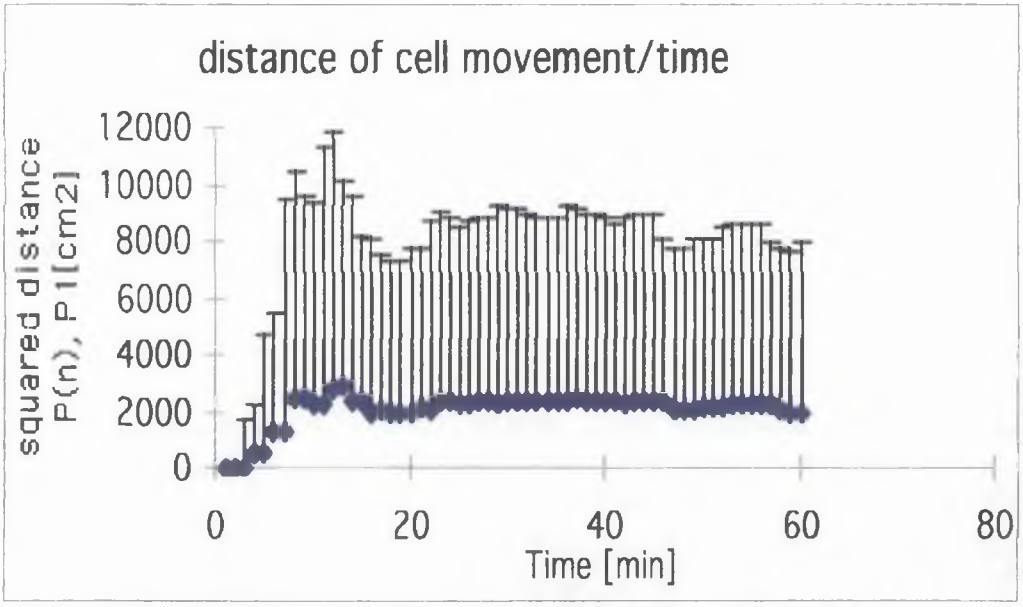


6. 4. Epitenon cells that were grown in 4%Dextran 503000

Figure 76:

There is a significant difference between the movement of epitenon cells grown in 4% Dextran and those grown in control ECT media as indicated by the curves below

a, *control*



experimental

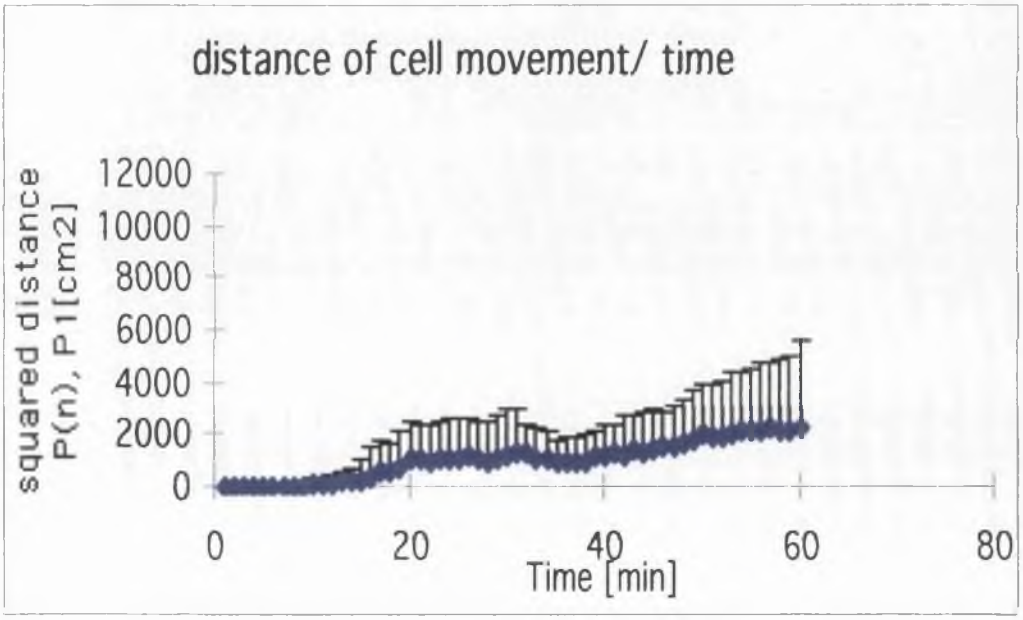
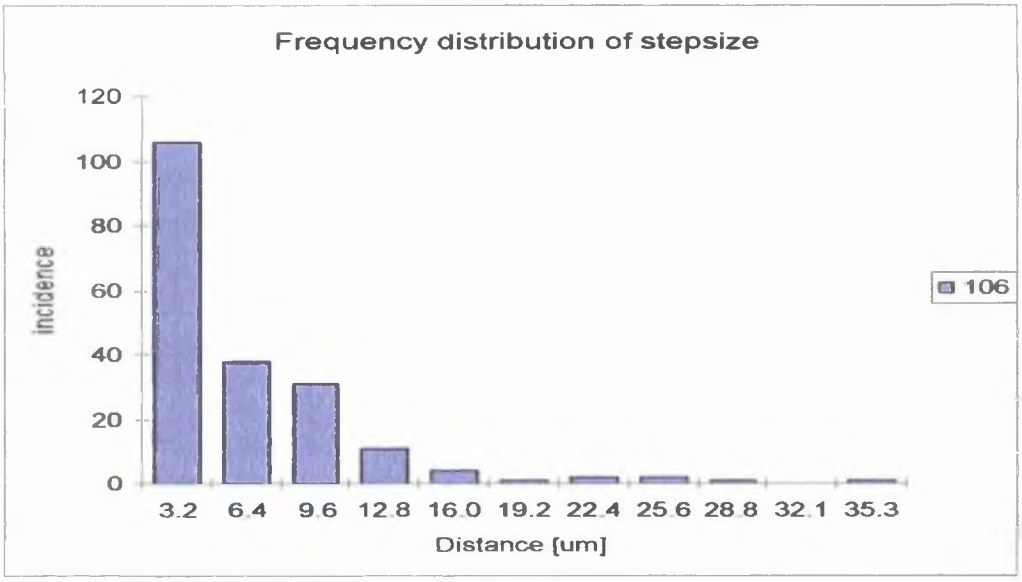


Figure 77:

There is a small change in the average step size of epitenon cells when cultured in 4%Dextran 503,000 compared with control cells.



control

experimental

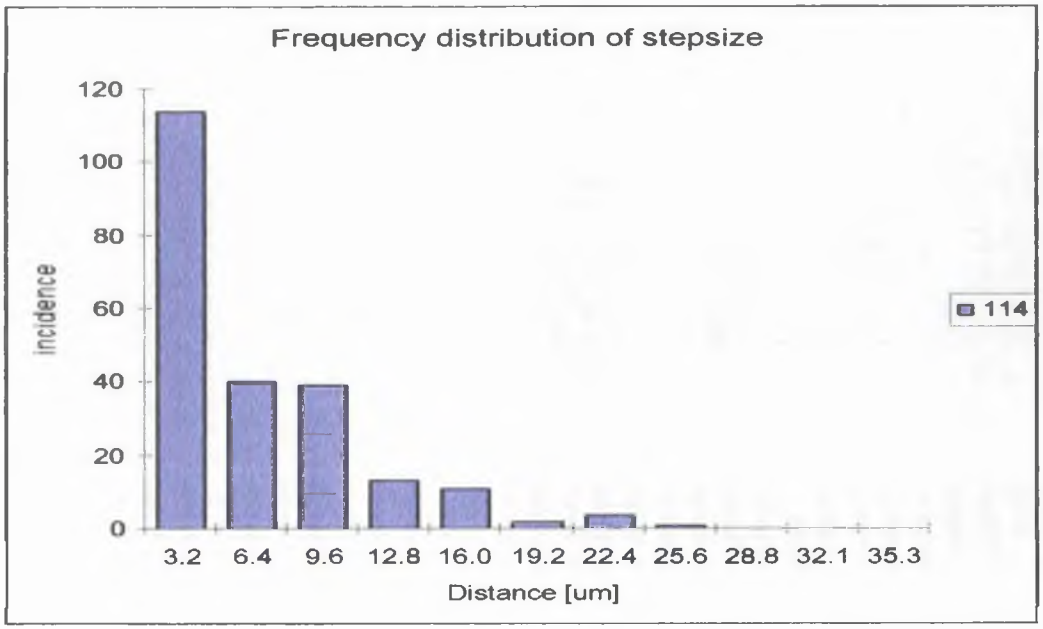
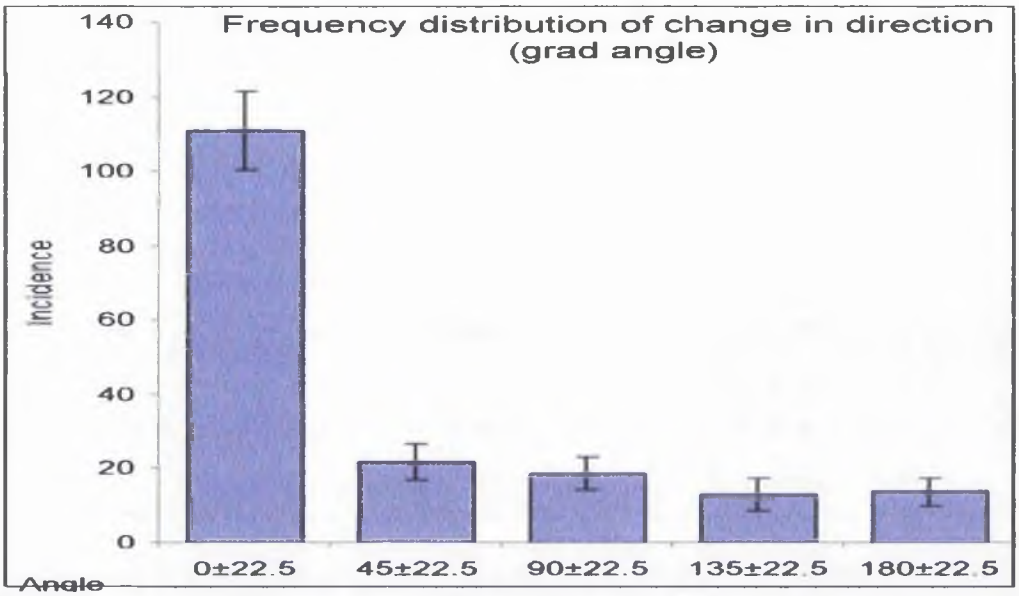


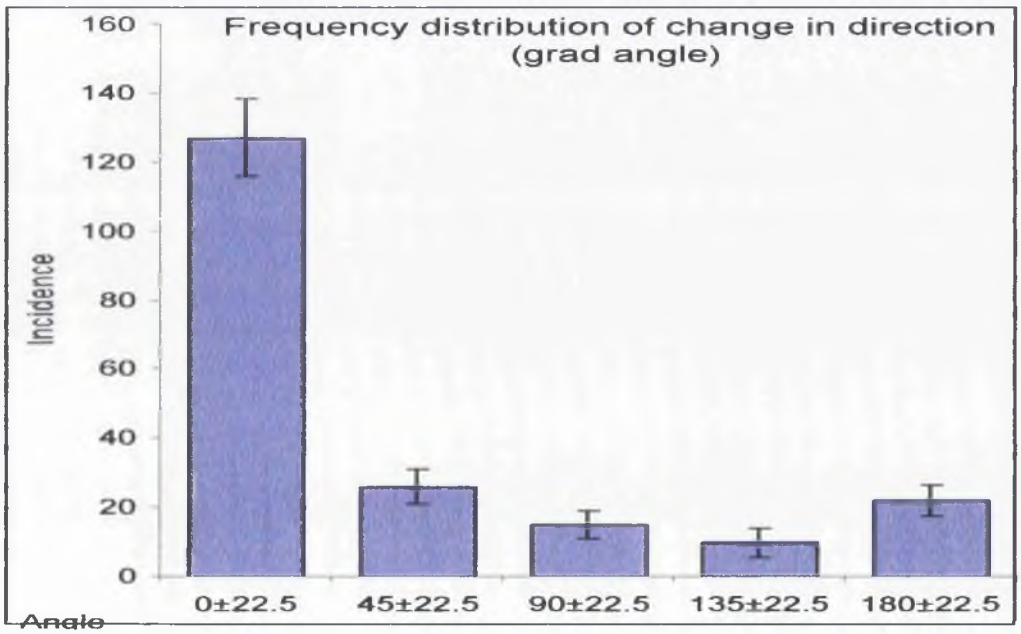
Figure 78:

The behaviour of epitenon cells cultured in 4%Dextran 503,000 shows little change from cells cultured in control ECT media as illustrated by the graphs below:



Control

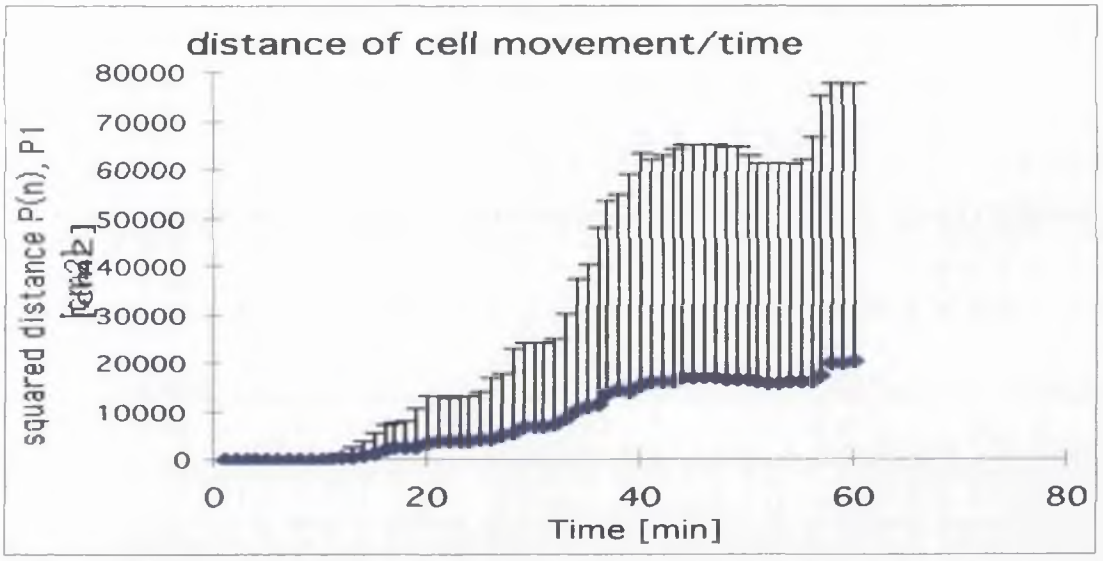
Experimental



6. 5. Epitenon cells in 8%Ficoll 400,000

Figure 79:

There is clear difference in the movement of epitenon cells when they were cultured in 8%Ficoll 400,000 compared with control cells in ECT medium, the movement curve of the cells drops sharply in the presence of Ficoll as is shown in the next graphs.



a, Control in ECT medium

b, *experimental*

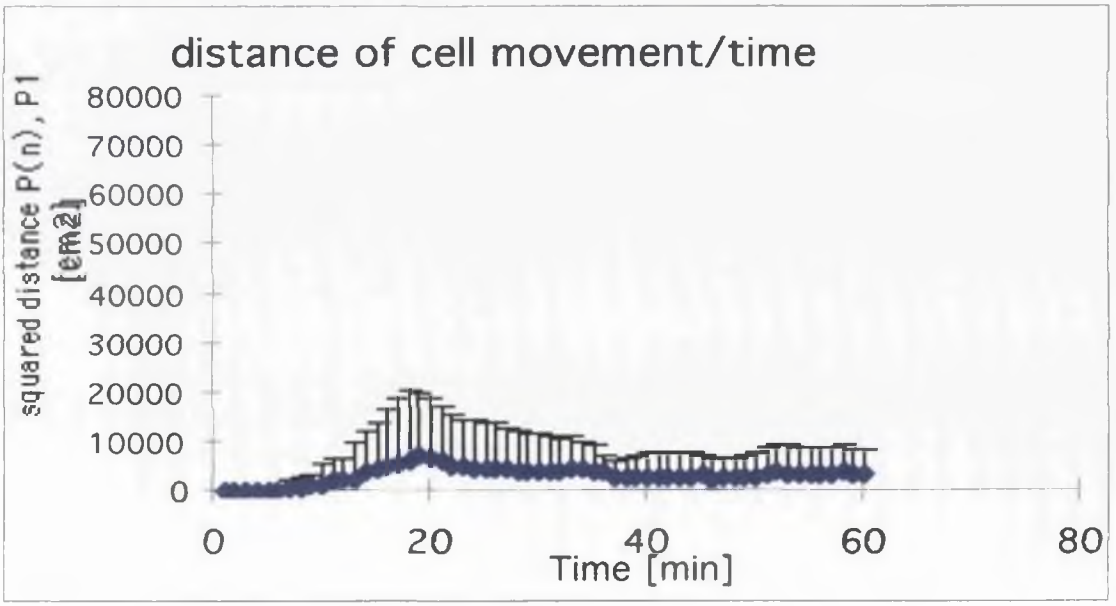
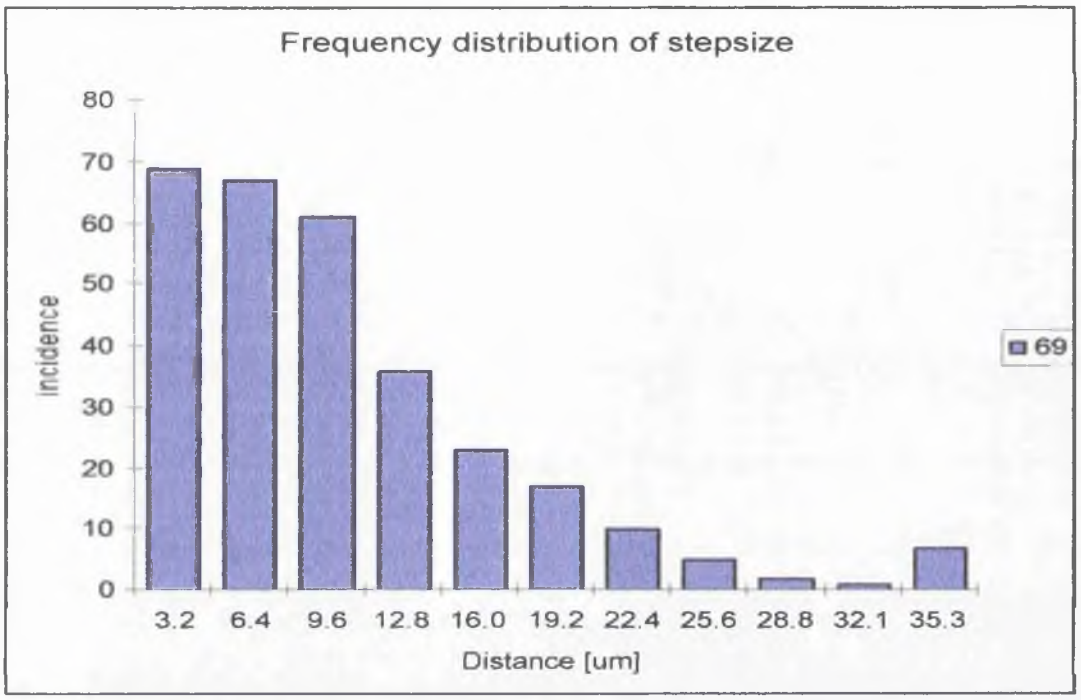
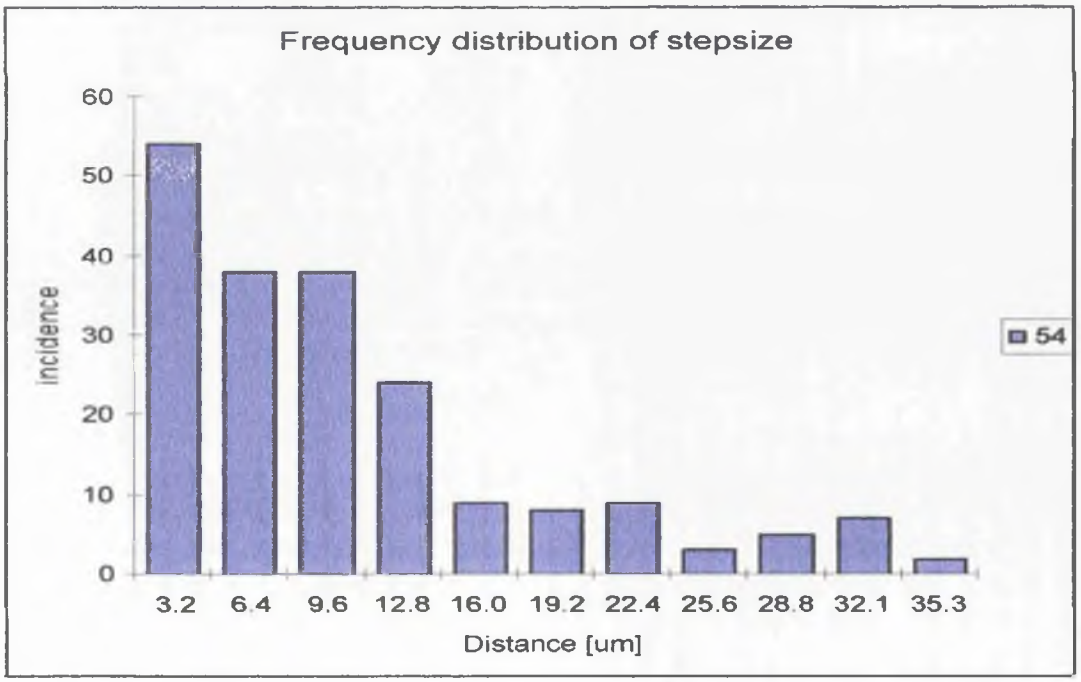


Figure 80:

There is a significant change in the average step size between epitenon cells grown in 8% Ficoll and those in control ECT medium, as is shown by the next graphs:



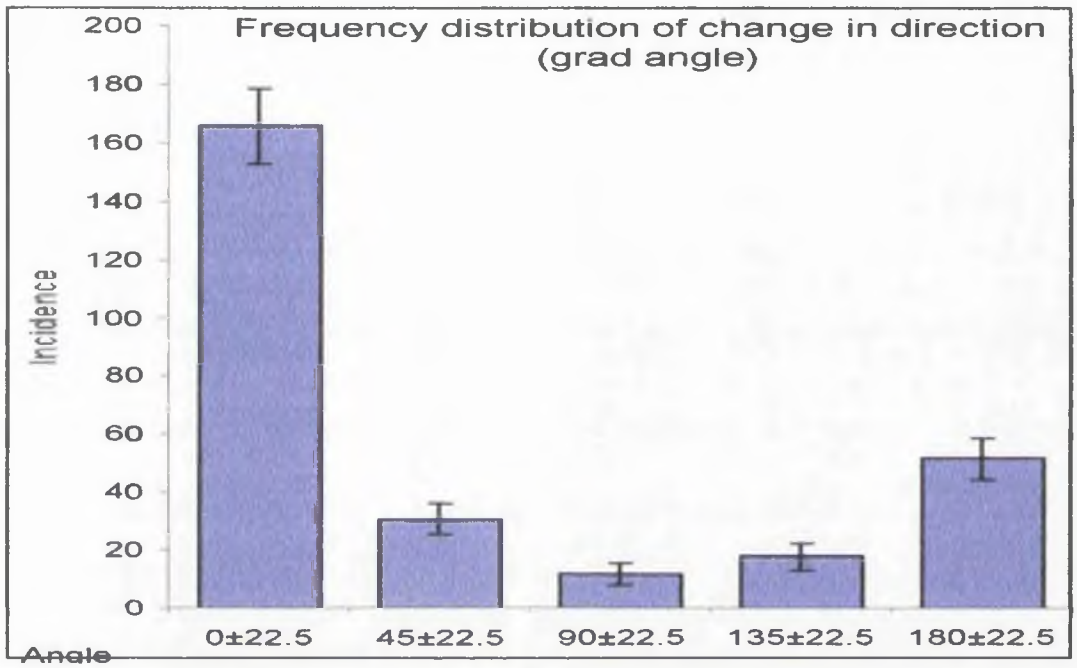
control



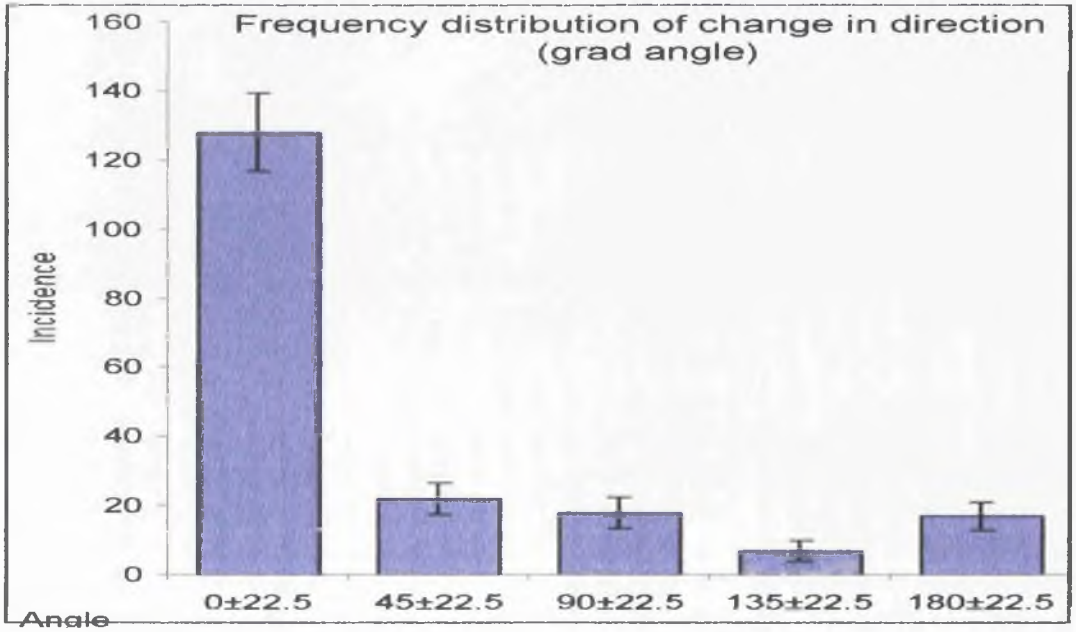
experimental

Figure 81:

There is a significant change of the direction of epitenon cells cultured in 8%Ficoll compared with the direction of control cells in ECT media, cells move in lose orientation.



control



experimental

Conclusion

From the experiments presented it can be deduced that while cells appeared to have extensive reduction in their movement in highly viscous media, after a time they can adapt to the new medium and start to move and migrate, though at a reduced rate compared with control medium. From this results I can conclude that cells are strong enough to move in a viscous conditions but if the viscosity of the medium increased too much (to 2.5 c.p.) cells can not adapt to it.

The effect of culturing cells in highly viscous media is to:

1. Reduce the movement of cells.
2. Reduce the average of step size of movement according to the distances.
3. Change the behaviour of cells (direction of movement) ie, reaction to structure.

The viscosities used were not sufficient to arrest movement but did reduce it especially in the case of endothelium. The cells clearly can exert sufficient locomotory force to the overcome viscous resistance.

In view of previous findings that the effect of high viscosity on the reaction of cells to topography increases with increasing concentration of the viscous molecules in the medium, it may be proposed that the cell size, shape and movement decreases with increasing of the viscosity of the medium.

7. CYTOSKELETAL STUDY

Experiment aims

The previous data in this thesis has indicated that the high viscosity media affect the cell morphology, adhesion and movement on adheesive surfaces.
This leads to the suggestion of a cytoskeletal reaction to medium of high viscosity.
Thus this was investigated.

Results

Morphometeric and cytoskeletal data will be presented for Epitenon and Endothelial B10D (2) cells on smooth and grooved surfaces.
On each surface, the distribution of actin microfilaments will be observed, after culture in control and high viscosity media for 24h, cells were fixed and stained with fluorescent phalloidin in order to examine the F-actin cytoskeleton (see Materials and Methods). The F- actin cytoskeleton was viewed by fluorescence microscopy
Results from these experiments show that actin morphology was affected by the high viscosity medium as explained below, see next figures 82 - 85.

1. As illustrated in the previous experiments, the Endothelial cells B10D (2) appeared to be more affected by high viscosity than the Epitenon cells (see 4.4 and 4.5). In this experiment also the cytoskeleton of Endothelial cells appeared to be less distinct than that of the Epitenon cells in high viscosity medium, see Figures 82-85 pictures 2 & 4 in each.
2. Cells were cultured on flat surfaces in control media, both Endothelial and Epitenon cell types were seen to be fully adhered to the surfaces with mature circumferential and stress fibers observed, but the cells were not polarised, see Figures 82 picture 3, 83 picture 3 and 84 picture 3.
3. Cells cultured on the flat surfaces in medium of high viscosity appeared to be very small in size especially the endothelial cells as mentioned before (Results 4.2). Only punctuated actin condensations, as observed in rounded-up cells were

seen in the endothelial cells (see Figures 82 picture 4 and 85 picture 4). The epitenon cells were seen to be more spread, but actin involvement in lamellipodia was commonly observed, as seen in spreading cells, compared to the controls where stress fibers were seen abundant in flattened cells (Figure 83 picture 4 and 84 picture 4).

4. The Endothelial cells cultured on grooved surfaces in control medium showed highly oriented actin fibers, whereas the cells cultured in very high viscosity medium (6% Ficoll 400,000 (2.26 cp.) or 4 %Dextran 503,000 (2.6 cp) on grooved surfaces showed severe effects in the distribution, loss of orientation and condensations of actin filaments (see Figure 82 pictures 1 & 2 and Figure 85 pictures 1 & 2).
5. Epitenon cells cultured on grooved surfaces in medium of high viscosity (4%Dextran 2000,000) or (4%Ficoll 400,000) showed some orientation and actin condensation compared with the fully oriented and well formed actin condensations in control cells, Figure 83 & 84 pictures 1 & 2.
6. Cells appeared to be affected by 6% Ficoll 400,000 (2.26 cp.) either on grooved or flat surfaces more than by 4%Dextran 2000,000 (4.56 cp.); The discrepancy can probably be attributed to differences in reagent chemistry.
7. In general these experiments suggested that the high viscosity of the medium could limit the actin microfilament assembly and operation, but not prevent it (see figures 82-85 picture 2 and 4 in each).

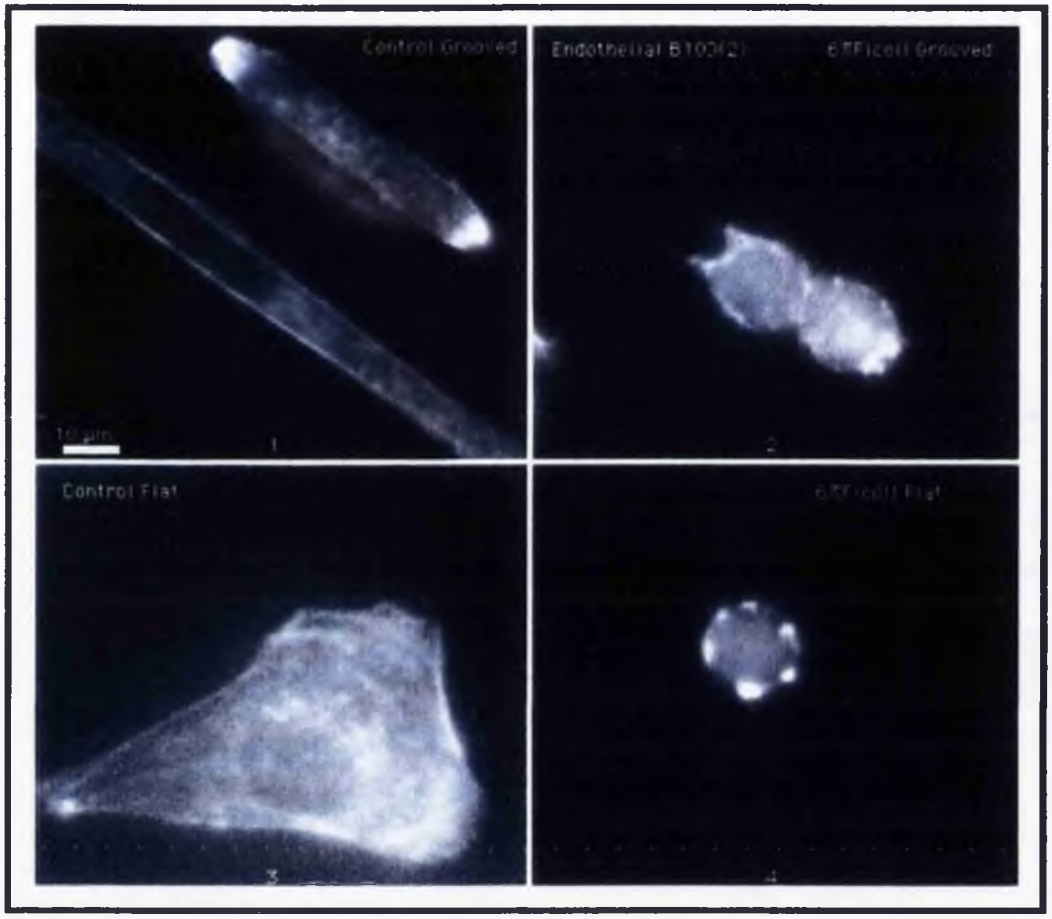


Figure 82:

Endothelial cells B10 D (2) were cultured in control Ham's F10 medium and 6%Ficoll 400,000 for 24h. Cells were stained with fluorescent phalloidin and examined with Fluorescence microscopy (Objective 50x):

1. Cells on grooved structure in control medium.
2. Cells on grooved structure in 6%Ficoll 400,000.
3. Cell on flat coverslip in control medium.
4. Cell on flat coverslip in 6%Ficoll 400,000.

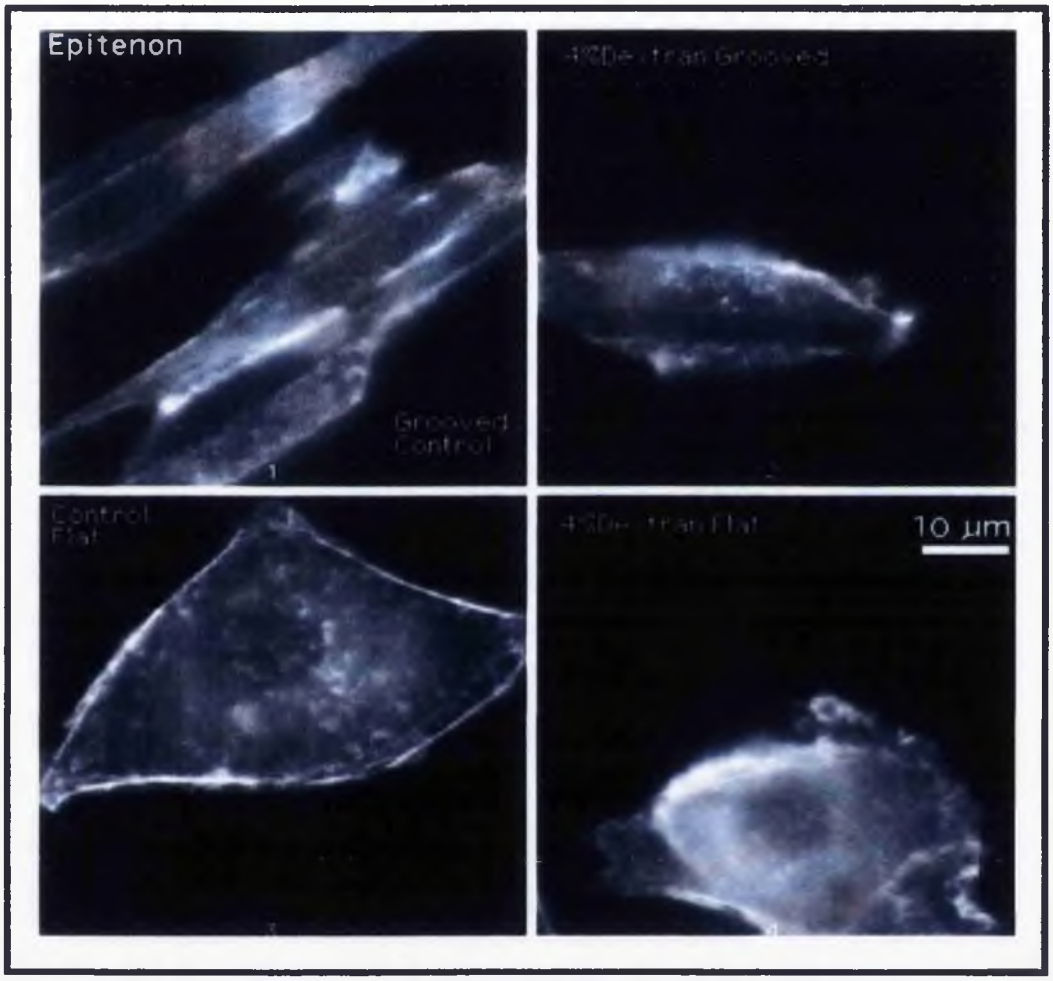


Figure 83:

Epitenon cells were cultured in control ECT medium and 4 % Dextran 2000,000 for 24h. Cells were stained with fluorescent phalloidin and examined with Fluorescence microscopy (Objective 50x):

1. Cells on grooved structures in control medium.
2. Cell on grooved structure in 4 % Dextran 2000,000.
3. Cell on flat coverslip in control medium.
4. Cell on flat coverslip in 4 % Dextran 2000,000.

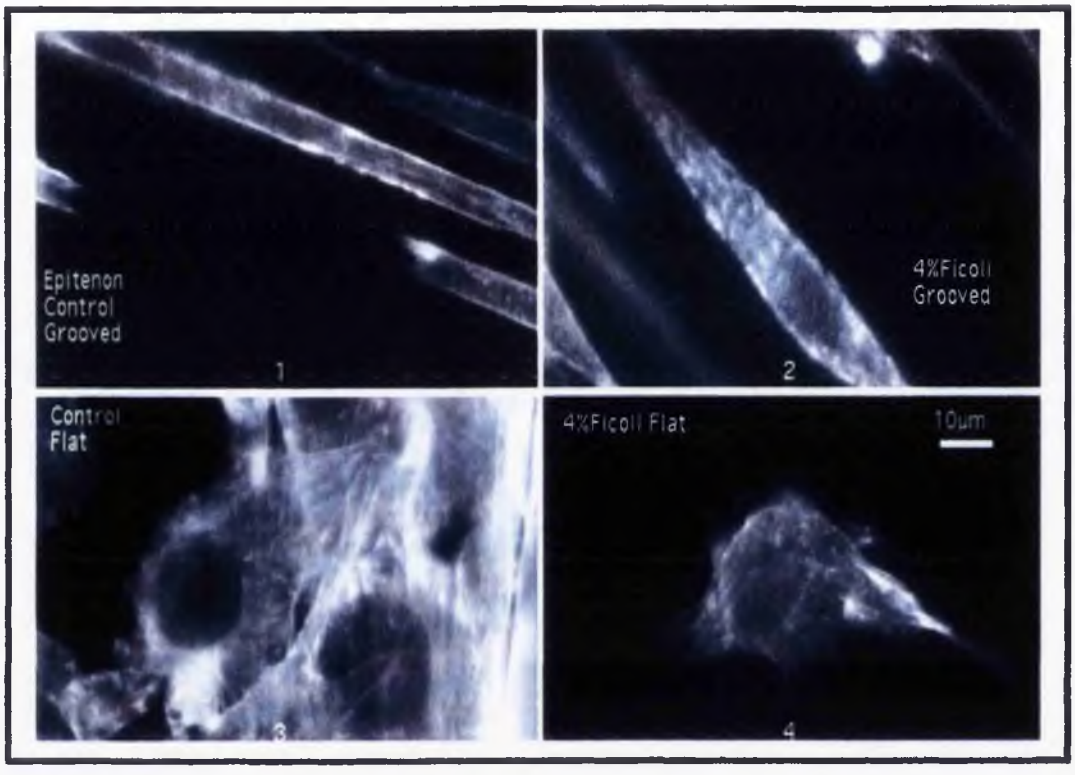


Figure 84:

Epitenon cells were cultured in control ECT medium and 4 % Ficoll 400,000 for 24h. Cells were stained with fluorescent phalloidin and examined with Fluorescence microscopy (Objective 50x):

5. Cells on grooved structure in control medium.
6. Cells on grooved structure in 4 % Ficoll 400,000.
7. Cells on flat coverslip in control medium.
8. Cell on flat coverslip in 4 % Ficoll 400,000.

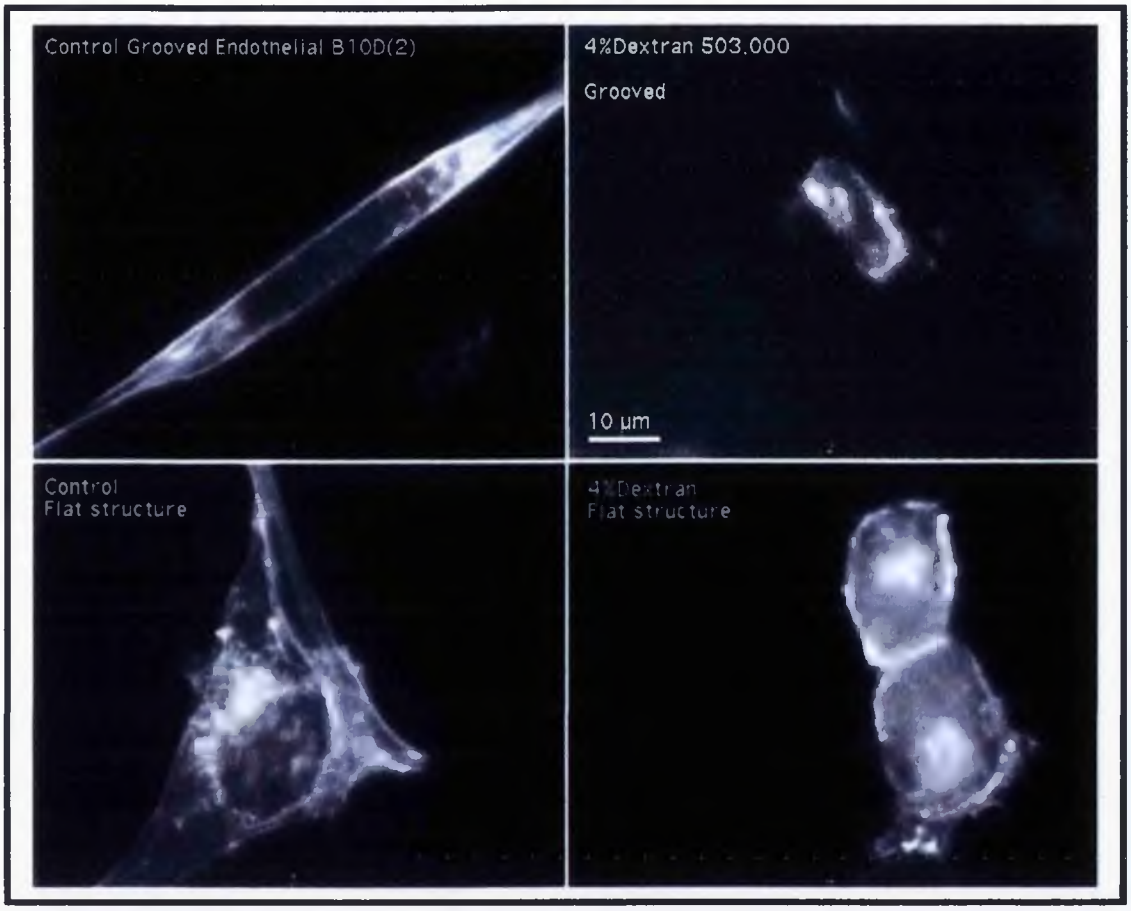


Figure 85:

Endothelial cells B10 D (2) were cultured in control Ham's F10 medium and 4%Dextran 503,000 for 24h. Cells were stained with fluorescent phalloidin and examined with Fluorescence microscopy (Objective 50x):

5. Cell on grooved structure in control medium.
6. Cell on grooved structure in 4%Dextran 503,000.
7. Cell on flat coverslip in control medium.
8. Cells on flat coverslip in 4%Dextran 503,000.

Table 19: Summary of all the effects of high viscosity on epitenon cells

Reagents name			Leg(μm)		IRM		Movement		Cytoskel	
C.p	Name	Co	Cnl	Exp	Grv	Flat	Cnl	Exp	Grv	F
0.75	ECT		93	-	N	N	-	-	OS/C	C
0.82	Dex 9K	2%		89	-	N	-	-	-	-
0.88	Dex 9K	4%		89	-	N	-	-	-	-
0.82	Fi c 70k	2%	87	87	-	N	-	-	-	-
0.94	Fi c 70k	4%		95	-	N	-	-	-	-
1.57	Dx503k	2%	81	70	S/D	S/D	-	-	-	-
2.58	Dx503k	4%		44	VS/MD	VS/MD	O/F	NO/R	OS	C
1.07	Dex 2m	0.5%	96	51	S	S/D	-	-	-	-
2.33	Dex 2m	2%		50	VS/MD	VS/D	-	-	-	-
1.57	MC	0.5%	120	69	-	-	--	-	-	-
3.08	MC	1%		68	-	-	-	-	-	-
9.37	MC	2%	81	40	-	-	-	-	-	-
<1132	MC	3%		10	-	-	-	-	-	-
0.82	Fic400k	0.5%	94	80	N	N	-	-	-	-
1.07	Fic400k	2%		59	S/D	S	-	-	-	-
2.26	Fic400k	6%	103	24.9	VS/MD	S/D	-	-	OS/P	P
2.58	Fic400k	8%		21.8	VS/MD	VS/MD	O/F	NO/R	-	-

C.p: medium viscosity, Leg (μm): average cells length, Cytoskel, Grv: grooved substratum, N: normal cell size and appearance, S: small cell size, VS: very small cell size, D: darker than control, MD: more dark areas in the cells, R: reduced cell movement, F: normal speed, O: oriented cells, NO: non-oriented cell movement, CD: condensation of the cytoskeleton, C: circumferential actin filaments, P: punctuate actin condensation, OS: oriented cytoskeleton, LO: loss orientation of the cytoskeleton, -: indicates no results.

Table 20: Summary of all the effects of high viscosity on endothelial cells

Reagents name			Leg(μm)		IRM		Movement		Cytoskel	
C.p	Name	Conc	Flat	Grv	Grv	Flat	Cnl	Exp	Grv	F
0.75	Ham's F10		100	178	N	N	-	-	OS/C	C
1.57	De503k	2%	-	128	N	S/D	-	-	-	-
2.58	De503k	4%	-	87	S/D	S/MD	O/F	NO/R	LO/P	P
1.07	Dex2m	0.5%	-	143	N	D	-	-	-	-
2.33	Dex2m	1%		95	S/D	S/D	O/F	NO/R	-	-
1.07	Fic400k	2%	-	114	S/D	S/MD	-	-	-	-
2.26	Fic400k	4%	56	50	-	S/MD	-	-	-	-
2.58	Fic400k	8%	10	13	VS/MD	MD/RU	O/F	NO/R	LO/P	P

C.p: medium viscosity, Leg (μm): average cells length, Cytoskel: cytoskeleton, Grv: grooved substratum, N: normal cell size and appearance, S: small cell size, VS: very small cell size, D: darker than control, MD: more dark areas in the cells, R: reduced cell movement, F: normal speed, O: oriented cell movement, NO: non-oriented cell movement, CD: condensation of the cytoskeleton, C: circumferential actin filaments, P: punctuate actin condensation, OS: oriented cytoskeleton, LO: loss orientation of the cytoskeleton, RU: cell rupture, -: indicates no results.

Chapter Five

Discussion

DISCUSSION

General Discussion

This project has studied the effects of the viscosity of the medium on the reaction of cells to topography. The reasons for doing this are to:

1. Consider the role of diffusion in cellular reaction to topography.
2. Look at cells in an environment resembling intercellular conditions where medium viscosity is fairly high.
3. Control the condition that permits the correct growth of cells in the laboratory first and then inside the body.
4. Control cell adhesion, position and orientation which are vital to give correct growth and cell function.
5. Study conditions (chemicals and topographical cues) that allow making constructs from living cells outside the body for subsequent implantation.

The medium viscosity has been changed by adding viscous macromolecules to the culture medium. The effects of viscosity appear to be linked to changes in cell morphology, spreading, movement and on theoretical grounds to mechanical interactions with the substrate.

Because high concentrations of solutes were added to experimental media, it must be a concern that physiological effects could be due to altered osmotic or chemical activity.

It is clear from the data that the contribution of the reagents used to the total osmotic pressure was small. Thus the osmolarity of the medium did not change too much after adding high molecular weight reagents. The fact that despite different chemistries the viscosity increasing reagents have similar effects argues that the chemistry of these reagents is not important in this study.

Thus viscosity but not colloid osmotic pressure is implicated in the response.

There may also be effects resulting from the extra force needed to extend a cell into a high viscosity medium, but I have no evidence on this as yet.

In this study, only two cell types were used to investigate their reactions in the presence of high viscous media. The cells demonstrated fairly similar reactions to topography. Both cell types can be affected by medium viscosity which results in changes of cell morphology and the rate of cell movement.

To understand the effects of medium viscosity we must first analyse the mechanisms by which viscosity alters cell morphology or movements.

Contact guidance refers to the reactions of cells with the topography of their substratum, as discussed in the introduction. The present investigations on the mechanism of contact guidance focus on the dynamic effect of high viscous media in relation to morphological and to changes of cell locomotion, as explained below.

Viscosity as a probe of cell reaction to topography

The major result of this study is that the morphological reactions to topography and the movement of cells on topography were both markedly slowed when the medium viscosity is increased, thus viscosity is a pertinent variable. The inverse dependence of reaction of cells to topography on viscosity strongly implies diffusional limitations to those reactions. As slower reactions were obtained for near-osmolar conditions of it is inferred that viscosity most likely affects the diffusional motions of molecules in and out of the space between cell and substratum. This in turn could nearly halve exchange rates compared with a cell in suspension and make exchange rates similar to those in the body. The results suggest that such diffusional motions need to be more carefully considered in experimental design of cell reactions to topography because it will affect the space between cells and topography.

Cells are able to orient themselves in response to external signals. The sensitivity of cells to signal gradients may be extremely high. Cell polarisation initiated by gradients of an external signal is then stabilised by global reorganisation of the cytoskeleton. Change in the external conditions of cells may be affecting the cell

polarisation by affecting the redistribution of pseudopodia due to the reorganison and orientation the whole cytoskeleton. Viscosity effects in narrow gaps on the outside of the cell may affect cell polarisation.

Variation of medium viscosity

In these studies the medium viscosities were altered by adding small concentrations of different macromolecules to the normal media (ECT and Ham, F10), such as Ficoll, Dextran, Methylcellulose and Carboxymethylcellulose.

Tuvia in 1997 and his group studied the effect of medium macroviscosity on cell membrane fluctuations, they modified the solvent viscosity by the addition of high concentrations of small co-solvents such as glycerol and sucrose, producing relatively high viscosity levels. They considered the possibility that the effect of macroviscosity on cell function is transduced through a direct effect of extracellular fluid macroviscosity on cell membrane dynamics.

Tuvia in his study, determined the medium viscosity by measuring its flow time, relative to water, using an Ostwald viscometer at 25° C. The same method was used in this study to measure the medium viscosity but at more relevant temperature (37^0C). He also used Dextran and Carboxymethylcellulose to increase the medium viscosity, the same macromolecules are being used in this thesis.

Chase et al in 1998, also used an Ostwald viscometer to measure the viscosity of fibre solutions relative to that of water in order to correlate directly muscle fibre mechanisms with bulk solution viscosity (at 12^0C). In their study relative viscosity (η/η_0) was calculated according to:

$$\eta / \eta_0 = tp / t_0p_0$$

Where t is the flow time measured in the viscometer, p is density, and the subcript 0 indicates measurements on water. They estimated the relation of viscosity and diffusion by the equation

$$D = kT/6\Pi\eta r$$

Where k is Boltzmann's constant and T is absolute temperature, r is the radius of a sphere and D is diffusion coefficient.

This is the same equation that is used to estimate the relationship between viscosity and diffusion in the present study.

Agreements and disagreements with other work

Previously many worker suggested that many types of cells react to microtopgraphy and nanotopography by changes in important cell behaviour processes including: cell morphology, adhesion, changes in movement, contact guidance, and tissue organisation (see Chehrouidi and Brunette, 1995; Brunette et al, 1988; Curtis and Clark, 1990; Singhvi et al, 1992), activation of tyrosine kinases (Nobes et al in 1995; Wojciak-Stothard et al, 1996), condensation of actin cytoskeleton (Rovensky and Samoilov, 1994; Oakley and Brunette, 1993), and changes in gene expression (Ohara and Buck, 1979; Oakley and Brunette, 1995; Webb et al, 1995; Meyle et al, 1994). Since it is easy to observe the morphology of the cell, most of the data here refers simply to morphology and its concomitant orientation.

Other workers suggest that cells do not conform to many topographies as closely as they would to planar surfaces (Dunn and Brown, 1986; Brunette, 1986; Clark et al., 1987; Curtis and Clark, 1990; Oakley and Brunette, 1993)). The reason for this could be that the cells are under tension between attachments to groove edges and that this tension pulls the plasmalemma away from the groove bottom.

Biological aspects of the IRM study

The results in the first part of the experiments here confirm that the morphological reaction of cells to topography is altered when the viscosity of the medium increases. The observation of cells reacting to topography using IRM in the experiments confirms that cells conform to planar surfaces more closely than they would to grooved topography.

According to Izzard and Lochner, (1976) and Verschuren, (1985) in IRM the intensity of white light, reflected at the underface of the cell, contains information about the closeness of contact between the cell and the substrate. For larger distances

of cell-substrate separation, the image appears as a uniform grey, as for the cell-free areas. The images of cells cultured in medium of high viscosity appeared to have darker grey regions than controls in normal medium, this indicates that the separation gap between cells and substrate decrease when the medium viscosity increases, differences in reflectivity having been corrected for the changes in medium refractive index.

The reasons for proposing a different explanation for the presence of darker grey areas observed in the cells cultured in medium of high viscosity are:

1. Possibly cells increase the number of close contacts, as described by Verschuren 1985, close contacts appearing as a broad areas of uniform low intensity (grey) reflection under moving cells or cell parts. Verschuren et al described in 1983 the formation of extremely large close contacts beneath mouse embryo fibroblasts after treatment with dipyridamole.
2. Verschuren concluded in 1985, that close contacts are associated with rapid cellular translocation, while cells in high viscous medium showed a decrease in the movement rate (see phase-contrast results).
3. There is also a possibility of the darkness in the IRM images is complicated by intercalation of molecules into the membrane.
4. It may be there is an increase in the number of focal contacts in the low motility cells in the present experiments. As Izzard and Lochner, (1976) and Abercrombie, (1980) found that focal contacts appear rather dark by IRM, which means that they are fairly close to the substratum. According to Fleischer et al., (1975) and Abercrombie, (1980), it may well be that focal contacts are merely distinctly formed components of the contractile and adhesive systems, which take shape within a more generalised background, perhaps along lines of tension where contraction is particularly resisted.
5. The IRM images of cells that were cultured in medium of high viscosity showed many dark fringes around the cells. In accordance with Gingell's (1981) theoretical calculations, he found a very dark fringe at the cell margin, which could not be explained by low cell-substrate distance, but is determined by the thin cytoplasmic lamella (70nm) with a gap of 30-50nm underneath it. If these

fringes were coloured, it could imply large cell-substratum separations but I did not see coloured fringes.

Effect of medium viscosity on extracellular matrix

The growth and development of an entire population society of cells could be controlled through structural alterations of extracellular matrix (ECM) that produce associated changes in cell shape and associated physical force redistributions (Ingber et al., 1981).

Ingber and Folkman (1987) stated that the extracellular matrix serves as a local "solid state" regulator of soluble growth factor action through its ability to modulate cell and nuclear structures. Another study from Machesky and Hall (1997) mentioned that adherence of cells to extracellular matrix mediated through integrins is essential for normal cell development and movement.

Madri and Williams in 1983, found that purified ECM molecules can modulate the growth and organisation of the capillary endothelial cells *in vitro.*

These suggestions seem important in the present study because the high viscosity medium could affect the thickness of the extracellular matrix. The matrix becomes reduced in thickness by high viscosity molecule interpenetration by adding or removing water and in turn affects the diffusion of molecules into/ or out of the cells and also could affect the adhesion of cells to the matrix, which is reflected in the reduction of cell movements.

Boubriak et al. (2000) found that the permeability coefficient of rabbit sclera of ECM decreases with an increase in molecular weight solute (10 and 40 kD dextran). This finding could possibly be explained by the increase in asymmetry of the molecular shape with increase in dextran MW. Dextran are random coil extended polymers that do not have well-defined configuration, it can however take on asymmetric configurations. The resistance to motion of asymmetrical particles is characterised by the frictional coefficients along the principle axes. Extended molecules would experience less resistance moving lengthwise than moving broadside, with the diffusion coefficient describing this 'end-on' movement dependent on the degree of

asymmetry of the particle. In free solution the diffusion rate of asymmetric molecules correlates predominantly with the long axis, because the molecules move randomly. In a matrix, an orientation effect causes restriction of broadside movement while offering little resistance lengthwise. The rate of diffusion of asymmetric molecules in a matrix appears to correlate mainly with the dimensions of the short axis, thus leading to an increase of diffusion rates in matrix in comparison with free solution.

It would therefore be anticipated that diffusion of solute through the ECM would decreases with increase of matrix viscosity.

Explaining the morphological reactions results

These studies (see chapter 4.2) have reported changes in cell morphology or shape when cells were cultured in medium of high viscosity. Cell length was decreased when the concentration of high molecular weight reagent increased in the incubated medium.

The IRM photographs and contour maps (see chapter 4.3. IRM) illustrate a series of findings which could be made out in all the photographs of the cells. The greater part of the cells cultured in control media lies 55 to 75nm away from the glass and the lesser part of the cells lies 35 to 55nm away from the glass. In comparison, in cells cultured in media of high viscosity, the greater part of the cells lies within 36 to 50nm away from the glass and the lesser parts of the cells lie 55 to 75nm away from the glass. This indicates that the separation distances between the cells and the substratum reduced with increasing the medium viscosity, which could be related to the resistance of the cells to the medium viscosity by adhering more to the substratum. Another observation from results is the decrease of pseudopodia number in the cells cultured in high viscous media. This leads the cell to adhere to the substratum by the cell body, this is in contrast to cells cultured in normal media which appeared elongated with well developed pseudopodia. In images of control cells more light regions indicate that the separation distance from the substratum

increased under the cell body and decreased at the edges of the cells near the pseudopodia as compared to experimental. Curtis reported in 1964 that in most cells the rest of the cell body has a narrow band of separation 10 to 20nm, running around the edge of the cell but in some very elongate cells with well developed pseudopodia this band is missing, presumably because the stretching of the cell has pulled the whole of the centre part of the cell farther away from the glass. He found that the greater part of the cell body lies 30 to 50nm away from the glass, which include the centre part of the cell. He also found that the front pseudopodia and the rear pseudopodia are the parts of the cell closer to the glass about 20 to 25nm away from the glass.

In many cells, the surface is folded into a series of parallel furrows and ridges which are about 2 to 5 μm wide, the ridges coming to within 25nm and the furrows being as far away as 50nm from the glass (Curtis, 1964). This pattern develops in cells of a more rounded form but has never been seen in elongate cells according to Curtis. This leads to an alternative suggestion, which is that cells in medium of high viscosity have more surface folds than those control cells which lead to increase the dark areas of the cells.

Explaining the movement results

One question, which has importance in this field, it is whether the cells that were cultured in medium with high viscosity move or not. Video observations of phase-contrast studies and live IRM studies indicated that cells still move in highly viscous media but at lesser rates than in control media. It seems that medium of high viscosity also affects the cytoskeletal structures. Which may introduce the rounded form of the cells incubated in highly viscous medium.

According to the conventional model of cellular locomotion, the extended lamellipodium or pseudopodium should adhere to the substratum, providing an anchor for the tractional force that draws the cell body forward. This tractional force might elevate a weakly adherent lamellipodium. If the forward edge of the

lamellipodium is not strongly enough anchored, then the tractional force, rather than drawing the cell body forward, could exert a torque that would swing the lamella upwards. The tractional force is likely to be generated by an actin-myosin interaction. But the ratio of the measured tractional force and the rate of retraction of the hinge can yield effective of viscosity (Elson et al, 1998).
Further support derives from the observations of Hunt et al. (1994), who demonstrated that increased solution viscosity slows and can actually stall microtubules translocated by single kinesin molecules in an *in vitro* motility assay.

This leads to the suggestion that the thickness of viscous media might affect the tractional forces of the cell protrusion which in turn affect the locomotion of the cell as a whole. Curtis et al. (1995), found that movement on grooved substrate is highly oriented, but not polarised and this is also seen during the movement of cells in high viscosity medium.

I conclude in summary that cells are able to locomote in medium of high viscosity on any surface (see movement study, chapter 4.5) and extend pseudopodia (live IRM chapter4.4, see movies in the supplemented CD), but locomotion is severely reduced, directionality is lost, and pseudopodia formation is severely defective. These results suggest a direct effect of medium viscosity on cell movements.

There were several routes by which cells might respond to high viscosity of the medium: (1) The first possibility is that cells shrink in present of thick medium as shown in the morphology experiments. (2) The second possibility is that cell membranes were ruptured in very high viscous medium. As shown in 8% Ficoll Fig 62, Live IRM Results. (3) The third possibility is that the physical state of the medium could affects the mechanical properties of the cells. This seemed likely since the movement of the cells affected by high viscosity of the medium, as shown by movement experiments.

Studies of the cytoskeleton

Activation of cells by reaction to topography is highly significant, and can be brought about by reactions such as the changes in cytoskeletal condensation produced by contact of a cell to topography (Oakley and Brunette, 1993; Wojciak et al., 1996). The role of cytoskeletal elements in contact guidance has been discussed by several authors (Dunn and Heath, 1076; Dunn and Brown, 1986; Brunette, 1986; Curtis and Clark, 1990; Wojciak-Stothard, 1995). However, the most widely accepted hypothesis on the mechanism of contact guidance (Dunn and Brown, 1986) is based on the role of actin microfilament bundles, not individual filaments.

Oakley and Brunette, 1993 examined the sequence in which microtubules, focal contacts and microfilament bundles become aligned to the substratum topography. They seeded human gingival fibroblasts onto grooved titanium surfaces as well as onto control smooth surfaces. They used fluorescent phallacidin to stain actin filaments. They found that focal contacts were closely associated with the termination of actin microfilaments and microfilaments bundles became aligned, as well as the cell itself, with the grooves.
Actin filaments (Dunn and Heath, 1979; Dunn and Brown, 1986; Oakley and Brunette, 1993) have been cast in major roles in the development of cell orientation and polarisation. However, it could be argued that the actin alignment might be a determinant of cell orientation.

Gunderson & Bulinski in 1988, mentioned the important role of microtubules for maintenance of cell shape and polarity of moving cell. Because of the interactions between cytoskeletal components of the cell, it is would be of interest to study the role of actin and intermediate filaments during reactions of cells to medium viscosity. In this study of cells spreading on grooved and smooth substrata over 24h period in medium of high viscosity, the distributions of actin filaments were examined by fluorescent microscopy.
In particular, ruffles and circumferential fibres were evident in cells on the grooved surfaces in control media where they clearly outlined the groove pattern.

In addition, actin condensations and orientation were specifically noted compared with loss of orientation and limited condensation of actin in experimental cells, but some actin still was observed at the cell edges. The overall shape of these cells (experimental) was generally circular without orientation to the grooves.
In addition, actin condensation and actin orientation along groove ridge transitions were seen in cells in high viscosity media even though the cell shape was unoriented.
According to a study by Wojciak-Stothard et al (1995), using confocal microscopy they were able to show the actin arrangements in BHK cells and demonstrat that actin condensation was related to the points where the cells contacted the groove wall, usually at the shoulder where the wall meets the ridge top. This observation supported the idea of the actin condensation at the cell edges in this study.
Rovensky and Samoilov (1994) showed that cells with circular actin bundles were prone to bending around a cylinder with much less pronounced orientation along its axis than cells with straight actin bundles. The reaction of cells to cylindrical surfaces was determined not only by the presence or absence of actin microfilament bundles but by their pattern in the cell.
Since the aggregation of actin was observed in cells cultured in medium high viscosity, this suggested that viscosity could alter the actin pattern in the cell. The actin pattern in the cell is affected by medium viscosity and this may be due to apparent stimulation of contact formation by changes in surface contact.
Because of the close relationship between focal contacts and actin microfilaments, it may these aggregations of actin in these cells affect cell adhesion as has been mentioned before in the IRM discussion.

The cells on smooth surfaces also became polarised and alignment remain random as stated by Dunn and Brown (1986) and Oakley and Brunette (1993).
In agreement with the earlier reports (Dunn and Brown, 1986; Oakley and Brunette, 1993), the cells entered and aligned within the grooves, and along the ridge.
Ohara and Buck (1979) described microfilament bundles aligned parallel to the cell axis, and they found that, unless the grooves influenced the orientation of the cell as a whole, there was no effect on the components of the cell. Brunete (1986) reported

that for cells aligned with grooves, alignment of all the cytoskeletal filaments reflected the orientation of the cell as a whole.

Curtis and Clark (1990), basing their suggestions on the reactions of cells to a wide range of topographies and noting the tendency of many cell types to ridge walk along sharp discontinuities, proposed that sharp discontinuities in the substratum caused actin condensation and that this in turn led to cell orientation.

Actin microfilaments bundles have been described as inflexible bundles linking to focal contact (Dunn and Heath, 1976, Ohara and Buck, 1979; Dunn and Brown, 1986; Dunn, 1991). This work suggests that high viscosity can limit their assembly and operation, which results in affecting the cell's locomotion in medium of high viscosity.

It can be concluded that the medium viscosity may affect the actin cytoskeleton and might tend to disrupt microfilament organisation. Disruption of actin filaments by high viscosity could reduce cell orientation on grating structures. In general terms that microfilaments contribute to the reaction of the cells to the medium of high viscosity.

General relevance to other aspects of cell biology

The topography around cells may be that of surrounding cells, intercellular materials or biomaterials. The reactions to topography include cell orientation, rates of movement, and activation of the cells (Curtis and Wilkinson, 1997). A few studies have reported that the changes in cell shape can influence the gene expression (Chou et al, 1995), therefore it would seem reasonable to investigate the effect of medium viscosity on gene expression.

Cellular responses to medium viscosity may share common mechanisms involving cellular morphology; movements and responses to mechanical force (Abercrombie and Dunn, 1975; Heath and Dunn, 1978; Brunette, et al, 1988, 1995; Elson, 1988; Curtis and Clark, 1990; Harris, 1994; Elson et al, 1998). For normal cells, this mechanism could play an important role during embryonic development and wound healing, allowing cellular proliferation or growth to be regulated in response to changes in chemical properties of the environment.

Possible sources of error

During the course of this project it was not possible to use the Total illumination when setting the TIRF microscopy this have been may due in the difficulty to reaching the critical angle of reflected beams.
TIRF microscopy could have confirmed IRM measurements.

As reported by Rohrbach in 2000, the electronic setup of TIRFM has two important tasks: first to control the deflection of angles and thus the evanescent illumination of the probes, second to collect the fluorescent light with the CCD-camera and a PC.
In the setting used in this study it may be that the difficulty was to collect the fluorescent light using CCD-camera through the specific filter-giving rise to a problem of correct alignment.

Further work

This study was the first report on how medium viscosity was affect the reaction of cells to topography, which includes the morphological, locomotory responses, and the mechanism of these reactions. It is reasonable to continue this study by looking at the effects on cell function at the molecular level.
Work also must be continued with TIRFM to track diffusion of molecules under cells in contact with planar and with grooved topographies.

It would also be of interest to investigate the morphological reactions in greater detail, using electron microscopy. This would allow observation of cell spreading and polarisation at high resolution.

The adhesion pattern seems to be determined by the locomotory state of the cells (Verschueren, 1985). However it is important to study the adhesion regions in the cells were they contact the substrate in the presence of medium of high viscosity

because they are firm attachments structures that hold the cell in place and in its spread shape. This could be done using immunofluorescence of adhesion related proteins, such as integrins or vinculin.

Physical properties contribution possibility, rule out the importance to examine these properties in the presence of highly viscous medium e.g. forces.

One of the practical considerations stemming from this work is that viscosity needs to be carefully considered in both the design of reaction to topography experiments and interpretation of experimental data.

Chapter Six

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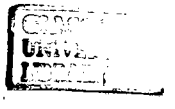

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Web Sites

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2. http://www.ks.uiuc.edu/Research/cell-motility/actin/.