ALIGNMENT OF RAT AORTIC SMOOTH MUSCLE CELLS BY HIGH ASPECT RATIO MICROFABRICATED ELASTOMERIC MICROCHANNEL CELL CULTURE SCAFFOLDS

by

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ABSTRACT

The desire for replacement organs, blood vessels, bone, and other body components is one of the many needs prompting research into tissue engineering. Engineering of functional blood vessels and muscle will require production of ordered cell cultures that orient cells preferentially to produce anisotropic deformation upon contraction. Toward this goal, this thesis investigated high aspect ratio microchannel cell culture substrates for their ability to align rat aortic smooth muscle cells in a high density cell culture. Two digital image processing methods were employed: two-dimensional fast Fourier transform used to evaluate cell alignment by measuring the orientation of F-actin filaments, and blob analysis to determine cell density and viability. Analysis of the orientation distribution of cells grown on the microchannel substrates, as well as cells in ex vivo tissue, provides the basis for determining the effectiveness of the substrate design for cell alignment. Based on the data obtained, microchannel substrates promote highly aligned smooth muscle cells, a necessary requirement for functional tissue, with up to 50% aligned within 10° of the channel axis. The density of actin and myosin associated with contractile cells was assessed using fluorescence quantification and immunohistochemical techniques, respectively, to determine the cell phenotype. The analysis of cell phenotype did not produce data consistent with a significant effect of the substrate on the cell characteristics measured. Ultimately, microchannel scaffolds may become an integral component of tissue engineering, working synergistically with other emerging techniques to promote functional tissue replacements.
APPROVAL FOR SCHOLARLY DISSEMINATION
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CHAPTER 1

INTRODUCTION

Need for Tissue Engineering

Tissue Replacement

Nearly every year sees an increase in the number of organ transplants performed in the United States, with the total amounting to 24,893 in the year 2002, according to data from the Organ Procurement and Transplantation Network (OPTN). Unfortunately, the number of people who die because they do not receive an organ transplant is also increasing annually. According to OPTN, administered by the United Network for Organ Sharing (UNOS), 6,366 people in the United States were removed from waiting lists for organ transplants due to deaths in 2002, accounting for 17.9% of total removals. As of October 10, 2003, UNOS reported a total of 82,580 U.S. patients were waiting for organ transplants. The situation is a result of the high demand for organ donation and the relatively low supply. As shown in Figure 1, the number of patients on waiting lists to receive an organ has been rising steadily over the last decade. As a result, for many organs, the mean time to transplant has increased over the past decade, as illustrated in Figure 2.
For most organs, the donor must die before the organs can be transplanted, with 73.9% of transplants performed in 2003 (as of July 18) using deceased donors. Only in a few situations, such as in kidney transplants, can living people become donors, though the trend is toward a greater percent of transplants coming from live donors as shown in
Figure 3. Even in these cases there are simply not enough organs to go around, partly because of the inconvenience and discomfort associated with the operation. In fact, 67% of people on waiting lists for organs are waiting for kidney transplants,\textsuperscript{5} and they account for over half the reported waiting list removals by death.\textsuperscript{2} In most situations involving living donors, the patient must rely on close relatives to be donors because people would be more likely to donate to a relative.

![Percent of transplants from living donors (1988-2002)\textsuperscript{1}](chart)

Compatibility between donor and recipient is another factor that complicates organ transplantation. In order to decrease the likelihood that the organ or tissue will be rejected by the recipient’s body, it is necessary to find donors and recipients who are compatible. An organ recipient’s immune system will view the new organ as an invader and attempt to kill it. If the donor and recipient tissues are similar, the immune system’s attack will not be as strong. This is another reason that potential transplant recipients
often rely on close relatives to donate kidneys. Even so, it is often necessary to suppress
the body’s immune system to prevent it from attacking the new organ.

Tissue engineering can potentially solve both these problems. Ideally, a sample
of tissue, from either differentiated cells from remaining functional tissue or stem cells,
could be harvested from the patient or compatible donor and be seeded into a three
dimensional scaffold implanted in the body. A tissue scaffold is essentially a physical
framework that provides cells with the requirements needed to allow the initial cell
population to increase in number so that final population of cells is appropriate in terms
of density and function. The form of the scaffold is as varied as the tissue it is designed
to support; however, most are geometrically complex, with a high surface area to volume
ratio, with surfaces that may be highly intricate and require special fabrication methods.
Scaffolds are designed not just for the physical support of cells, but also to provide for
introduction of nutrients and removal of cellular wastes. Other tissues such as blood, for
example, may not even need such a physical scaffold. The scaffold’s surface chemistry
and microstructural surface features work synergistically to induce proliferation of cells
which fill the scaffold, and differentiation of cells to provide the necessary function of the
desired tissue.

Because a small tissue sample could be used from which to grow the organ
needed, live donors could be used, and using the patient’s own cells to grow the
engineered tissue eliminates the need for donors entirely, solving the problems with
rejection of donated organs. Implantable scaffolds should be manufactured from
materials that naturally degrade in the body so that the scaffold does not need to be
removed, allowing for the engineered tissue to fully incorporate into existing tissue.
Other Motivations

**Cell-based Bioassay Systems.** There are motivations for developing tissues for purposes other than organ replacement. One area of potential use would be as a bioassay system that incorporates live cells. Such systems show promise for detecting chemical toxins and pathogens. They have advantages over traditional molecular based assays, since unlike molecular assays, no prior knowledge is necessary of the mechanism of interaction of the toxin with the cell. One type of tissue that is of interest for these devices is an engineered liver tissue, consisting of hepatocytes. These cells were chosen because hepatocytes should be sensitive to toxins, as their removal is their primary function. Alternatively, a large array of different cell types could be included, and the overall health of the cells monitored.

**Drug Discovery.** Drug discovery and testing is another area that can potentially benefit from the production of artificial tissues. Due to the expense of drug discovery, steps are taken early in the process to weed out ineffective compounds by solution binding assays. It has been shown that drug interactions outside of a living tissue may be different than that experienced \textit{in vivo}. For this reason, \textit{in vivo} models are used, but their cost greatly increases the expense of pharmaceutical development. A more economical solution is to use cell-based testing to determine the most promising lead compounds for \textit{in vivo} testing. It must be noted that traditional cell culture systems are poor predictors for drug testing results since their phenotype, the essential characterization of their function, is easily modulated by the artificial culture environment. To truly reap the benefits of cell-based drug discovery, tissue culture systems must be developed to allow control over the cellular microenvironment, producing models of animal tissues that are
more economical and more uniform than \textit{in vitro} models currently used in the pharmaceuticals industry, yet still similar in behavior to \textit{in vivo} cells.

\textbf{Properties of Ideal Engineered Tissue}

Engineered tissue should have as many traits in common with \textit{in vivo} cells as possible. Ideally, the engineered tissue should function identically to an \textit{in vivo} cell. Tissue function is related to its structure on the tissue, cellular, and molecular levels. Tissue structure is a combination of the types of cells present in a tissue as well as their respective organization within the tissue. In general, functional tissue cannot be perfectly replicated with only one type of cell. For example, replicating an artery requires smooth muscle cells and endothelial cells. In order to engineer tissue, however, it might first be preferable to simplify the tissue to reduce the complexity of interactions among the many variables. For study purposes, it might be desirable to create a muscle tissue with only one type of cell, i.e. smooth muscle cells, whose constituents could be studied separately from the complicating interactions of other cell types.

Simply culturing cells will not create a tissue; the organization of these cells within the tissue is also important. Cells must be induced by some method to orient themselves in the formation of an \textit{in vivo} tissue. For muscle tissue to function properly, the muscle cells must be oriented along a common axis. This will allow the bulk muscle tissue to contract upon stimulation. In standard cell culture, cells orient themselves randomly, resulting in a group of cells that are incapable of contracting in unison to produce anisotropic geometrical deformation. Furthermore, simply having cells that are
aligned with each other is not sufficient for full functionality. The cells must also form contacts so that a single contractile impulse will travel throughout the tissue.

There is a lower level of structure to which tissue function is related—cellular structure. For a muscle cell to contract, it should have high quantities of actin and myosin in filaments oriented to allow cellular contraction. These are qualities of a cell’s phenotype, and are useful indicators of a cell’s function. A muscle cell in culture with low myosin levels will not be highly contractile, but will be more likely to proliferate than a cell with high myosin levels, which is capable of being highly contractile. Thus regulating a cell’s phenotype is important to ensure optimal functionality of the bulk tissue.

Another aspect of cellular structure is the three dimensional shape of the cell. A cell grown on a standard cell culture substrate will adhere to only one surface. This results in a flattened cell. For a more physiologically natural cell shape, the cell must make attachments in three dimensions. It is known that the shape of a cell can affect its function.\(^8\) Therefore, to produce a fully functional tissue, it is desirable to influence the cell shape to emulate that of an \textit{in vivo} cell.

\textbf{Background Information}

\textbf{Smooth Muscle Cell Biology}

Smooth muscle cells (SMCs) are found primarily surrounding vessels in the body like blood vessels, such as the aorta, or intestines. Compared to skeletal muscle, they respond much more slowly to contraction and relaxation stimuli. Unlike skeletal muscle, which may contain hundreds of nuclei, smooth muscle cells are mononucleate. The
structure of the smooth muscle cell is much less organized than its skeletal counterpart, with the filaments being associated in loose bundles. Z-disks are absent from SMCs, with the filaments being attached to dense bodies instead. The filaments connect to attachment plaques which are the points at which the generated force is exerted. These focal adhesion plaques contain large amounts of the proteins vinculin and α-actinin, which serve to connect the filaments to either a substratum or to neighboring cells.

Despite the differences between smooth and skeletal muscle, there are many similarities, with the force generation methods being equivalent. Briefly, muscle cells have two types of filaments: thick and thin filaments. Thick filaments are rich in myosin, whereas the thin filaments are primarily made of actin. According to the sliding filament model, adenosine triphosphate (ATP) interacts with myosin and actin, generating a force that causes the thin filaments to slide along the thick filaments. This is initiated by a rise in the Ca\(^{2+}\) levels, primarily entering through the plasma membrane channels. A variety of smooth muscle-specific proteins exist that function in the role of muscle contraction. While troponin plays a central role in muscle contraction in skeletal muscle, this protein is absent in smooth muscle. Instead various other proteins fulfill its role, such as caldesmon, an actin-binding protein that is activated at low Ca\(^{2+}\) concentrations, preventing muscle contraction. Filaments are not limited to the contractile apparatus of the cells, but they are also found as stress fibers which are integral components used in the cells adhesion to its substratum.
Regulation of Smooth Muscle Cell Phenotype

There are essentially two distinct phenotypes exhibited by smooth muscle cells: the contractile phenotype capable of muscular contraction, and a proliferative, synthetic, phenotype that is highly mitotic. The contractile phenotype is the dominant phenotype expressed by cells in mature animals. The proliferative phenotype is predominantly found in organisms in their developing stages; however, there are instances, such as in wound healing and vascular remodeling, where proliferative cells are found in mature organisms. Thus, to achieve the goals of tissue engineering with SMCs, it is necessary to regulate the cell phenotype, causing cells to divide to increase the cell density of the engineered tissue, and then to cause the cells to cease division and become contractile. While proliferative cells can switch their phenotype to become contractile in vivo, it is also essential to study how and by what methods cells can dedifferentiate—that is, to become proliferative. To adequately meet the goals of tissue engineering, it will be necessary to control cellular differentiation: first to encourage proliferation to increase cell numbers, then to encourage differentiation to provide a functional tissue. Fortunately, it has been shown that contractile cells can be made to switch phenotype and become proliferative, and that this change is often reversible. The next issue to be addressed, then, is to describe the attributes of contractile and proliferative cells and how they are regulated and measured.

The Proliferative Versus the Contractile Phenotype. The proliferative phenotype is characterized by a number of attributes: cell division, production of extracellular matrix (ECM) components, and lack of contractile ability. On the other hand, the
contractile phenotype is primarily dedicated to contraction and, as a result, exhibits decreased rates of cell division and ECM production. Analysis of the phenotype of smooth muscle cells is also made difficult by the cells’ natural tendency to modulate from a contractile to a proliferative state. Furthermore, contractile cells have been shown to be capable of dividing, proving that it is not necessary for a contractile cell to dedifferentiate to become proliferative. A variety of factors influence cell phenotype including cell-cell interactions and the extracellular matrix.

**Cell-Cell Interactions.** There are many factors that affect cell phenotype, some of which involve the interactions between the cells of a tissue. Cells do not exist in isolation, as their interactions with other cells of both the same (homotypic) and different types (heterotypic) contribute significantly to their overall function. For a muscle tissue to be capable of contraction, its constituent cells must physically interconnect with other cells. Smooth muscle cells form attachment plaques that bind themselves to one another so that the contraction of all the cells in the tissue causes a dimensional change in the tissue, as shown in Figure 4 and Figure 5. A high seeding density has also been shown to have a significant effect on preserving the contractile phenotype. Contact inhibition is a phenomenon related to the behavior of cells when contacting other like cells. Typically, cells cease division and movement on contact with neighboring cells, resulting in a cessation of proliferation upon confluence. Heterotypic stimuli exert a profound influence on SMCs as well. It is known that endothelial cells are important in the regulation of smooth muscle cell proliferation by production and secretion of factors such as platelet-derived growth factor (PDGF). Neurons have also been shown to control smooth muscle cell phenotype *in vivo*, since cells deprived of normal neural input have
demonstrated a decrease in contractile ability.\textsuperscript{10} Despite all that is known, a large number of cellular interactions probably are still to be discovered, particularly at the level of molecular signaling.

![Diagram of contracted smooth muscle cells](image)

**Figure 4. Diagram of contracted smooth muscle cells**

![Diagram of relaxed smooth muscle cells](image)

**Figure 5. Diagram of relaxed smooth muscle cells**

**The Extracellular Matrix and Integrins.** The extracellular matrix (ECM) is very important in regulating the cell phenotype. Although much of what is known about the role of the extracellular matrix is in regard to fetal development,\textsuperscript{10} the ECM has very important regulatory functions as well. A wide variety of ECM components, such as collagen, elastin, laminin, fibronectin, glycosaminoglycans, and vitronectin, have been used for study of \textit{in vitro} cultures.\textsuperscript{10,14,15} These proteins interact with molecules on the surface of the cell, such as integrins, which determine the cell’s response.\textsuperscript{14}

Integrins, receptors present on the cell membrane, play a critical role in regulation of cell behavior, and they are responsible for both adhesion and signaling. Cell adhesion is accomplished by binding integrins with various ECM components surrounding the cell, as shown in Figure 6. However, a potentially more important role of integrins is to act as
signaling receptors, conducting information both into and out of the cell, controlling proliferation, function, and viability. Of the larger set of extracellular matrix proteins found in the body, a small subset is normally present in smooth muscle cells. This collection of ECM components changes with the cell’s phenotype, by either being the stimulus or the product of the phenotypic change. ECM components found in normal smooth muscle cells, in at least moderate amounts, are elastin (capable of inhibiting modulation of SMCs to a proliferative phenotype), laminin, fibronectin, vitronectin, various proteoglycans, and collagens type I, III, and IV.17,18

Figure 6. Diagram of molecules used to attach cells to extracellular matrix and other cells (Adapted from Lodish et al.)19

Phenotypic Differences Between \textit{in Vivo} and \textit{in Vitro} SMCs. A significant amount of data has been accumulated concerning the modifications cells undergo when cultured \textit{in vitro} as compared with the \textit{in vivo} environment.10 Namely, the contractile
ability of the cells steadily declines in direct correspondence with the decrease in contractile proteins such as actin and myosin. It has been demonstrated that production of actin and other proteins ceases immediately upon introduction into an *in vitro* culture.\textsuperscript{11} A number of factors affecting SMC phenotype modulation were recently summarized by Owens.\textsuperscript{10} These include procedural factors such as cell isolation and passaging methods, seeding density and cell-environmental parameters such as media serum, cell animal species, and substrate properties.\textsuperscript{10} With so many factors having synergistic effects, it is difficult to perform accurate comparisons between cells of *in vivo* and *in vitro* cultures.

**Phenotypic Markers.** A wide range of proteins are expressed in smooth muscle cells that are characteristic of the contractile state. The most important of these is smooth muscle α-actin, which accounts for more than 40% of the total cell protein.\textsuperscript{20} Although it has been shown that the ratio of the different smooth muscle actin isoforms (smooth muscle α-actin, nonmuscle β-actin, nonmuscle γ-actin, and smooth muscle γ-actin) may be definitive of cell phenotype, it has been suggested that the total amount of smooth muscle α-actin may actually be more useful for this determination.\textsuperscript{10} Another essential component of a functional muscle cell is myosin. Specifically, smooth muscle myosin heavy chains (SM MHC) are currently considered to be the best indicator of contractile smooth muscle cells, as they are believed to be expressed only in contractile SMCs, to the exclusion of proliferative SMCs and other cell types.\textsuperscript{10}

Smoothelin is a cytoskeletal protein that has been shown to colocalize with smooth muscle α-actin.\textsuperscript{21} Although originally postulated that it was found only in contractile smooth muscle cells and thus a suitable phenotypic marker for SMCs,\textsuperscript{22} its
presence is detected early in fetal development,\(^{23}\) casting doubts about such claims. Despite these reservations, it appears to be highly sensitive to modulation of cells, with expression of smoothelin ceasing before the shifts from desmin to vimentin expression, and from $\alpha$ to $\beta$ isoforms of actin.\(^{24}\) As it has been isolated much more recently, it has been less thoroughly studied than either actin or myosin.

The ratio of desmin to vimentin has also been claimed by many to be a useful marker of contractile SMCs. This is because of its increased expression in muscular arteries compared to those naturally more elastic,\(^{25}\) and its low values in cells induced to proliferate after vascular injury.\(^{26}\) There are a variety of other potential phenotypic markers such as $h$-caldesmon, calponin, vinculin, metavinculin, tropomyosin, and integrins such as $\alpha_1$-integrin, although many of these additional proteins have been much less studied than actin and myosin.\(^{10,27}\)

**Cell Morphology**

The morphology of cells can dramatically influence a wide variety of cell qualities such as proliferation rates, phenotype, and cytoskeletal properties.\(^{28}\) The morphology of cells differs significantly in cells cultured in an *in vitro* environment, as opposed to those found *in vivo*. Most noticeably, cells become less elongated and spindle shaped, and more polygonal, with focal adhesions formed in many directions. This is associated with the orientations of actin filaments within cells that are oriented along one axis in cells found *in vivo*, but become randomly oriented in the *in vitro* culture environment. Actin filament orientation is a major component of cell morphology that is clearly related to cell function. Another easily measurable aspect of cell morphology is nuclear elongation, or its inverse, nuclear roundedness. Controlling cell and nuclear
shape allows assessment of the effect of these measurements on cell function. Various measures of nuclear shape are possible, such as ratio of length to width (elongation) and area to height. A wide range of properties of cell morphology can be quantified. However, the relationship between these properties and cell function are not always well understood.

**Cell Culture Surfaces**

**Biocompatibility.** As mentioned above, the cell culture surface interacts with adherent cells by a variety of means, both mechanical and chemical. Because many of the mechanisms by which substrates affect the viability and phenotype of cells are unknown, substrates are often categorized by their “biocompatibility.” The term biocompatible has often been applied to materials without reference to their application, resulting in misconceptions about which materials are, and which are not, suitable for various purposes. Materials that prevent protein adsorption or cell adhesion may be seen as highly biocompatible in situations where this is desirable, such as for the prevention fibrous encapsulation, whereas these same substrates, when applied to tissue engineering, may be highly incompatible with cells if they are designed as cell scaffolds. Furthermore, some tests for biocompatibility test only whether materials placed in cell culture media cause a decrease in the viability of cells grown in that media, but have no physical contact with the material. This ignores important factors such as the cell’s ability to attach to a surface. Here, the important effect is intrinsic surface toxicity (IST), defined as the percentage of dead cells as measured by trypan blue inclusion. This theoretically should take into account all factors influencing cell viability as related to cell culture.
A number of factors can affect the toxicity of a surface to cells. Cytotoxicity, caused by chemicals toxic to cells leaching from a surface and killing them, is one known cause of a lack of biocompatibility. Another factor involved in cellular health is the surface charge of the substrate. Cells need at least a moderately charged surface to attach,\(^{30}\) and generally prefer negative-potential surfaces.\(^{31}\) However, neither wettability nor a lack of cell attachment has been found to correlate well with cell death.\(^{29}\)

It has also been found that protein adsorption, in addition to that spontaneously deposited on substrates in the presence of cell culture media, can be extremely beneficial in improving the cell viability.\(^{14,29,32,33}\) Conceivably, in addition to aiding adhesion, these proteins could be used as a physical barrier to the harmful effects of a substrate, since it has been found that in multilayer films, only the outer film layer affected the growth of cells on the surface.\(^{34}\) However, it has also been shown that cells still detected proteins embedded in up to 25 layers, though this effect lessens as more layers are deposited and may be the result of voids created by poor layering above proteins.\(^{35}\) A low cell seeding density has also been shown to lead to a high percentage of cell death on some materials, with the percentage of dead cells drastically reduced on densely seeded cultures.\(^{29}\)

**Surface Energy.** A wide variety of data exists regarding the optimal surface energy for cell attachment. In general, hydrophilic (high surface energy) substrates adsorb cells more readily and resist protein deposition,\(^{36,37,38}\) while hydrophobic (low surface energy) substrates are prone to protein adsorption and repelling cell attachments.\(^{38}\) However, some serum proteins such as fibronectin and vitronectin have been found to adhere to hydrophilic substrates and may be the cause of increased adhesion on wettable surfaces.\(^{39}\) Because cells attach to surfaces via proteins, either a
wettable or non-wettable surface can potentially be suitable, provided that adequate protein is first deposited on the hydrophobic surfaces to make them more hydrophilic.\textsuperscript{38,40} To provide “cell repellant” areas, serum-free media should be used. Cells attach to hydrophilic surfaces more readily because the higher surface energy increases the ability of the cells to form focal adhesions to the surface.\textsuperscript{41} Furthermore, it has been demonstrated that cell metabolic rates increase with wettability, suggesting that cell function is enhanced by factors other than increased adhesion.\textsuperscript{38} Data from several cell types have shown that the optimal contact angle is one that is moderately hydrophilic, with gradually decreasing adhesion at higher and lower surface energies.\textsuperscript{42}

Although chemical modification can alter the surface energy, there is also some evidence that it can be increased by certain surface topographies. It has been reported that microchannels with 5 μm width and depth reduce the surface energy of silicone substrates as measured by a decrease in contact angle.\textsuperscript{43} However, other researchers have reported no change in wettability and surface energy using substrates of similar surface structure.\textsuperscript{37} In general, wettability correlates with adhesion, but not with toxicity to cells or cell death.\textsuperscript{29} Further investigations have determined that, on some substrates at least, cell proliferation correlates better with oxygen groups present on the surface of a substrate rather than with wettability.\textsuperscript{40} For the most part, cells are generally attracted to hydrophilic surfaces, so mechanisms to control this aspect of a substrate are crucial, although surface energy is only one of many relevant surface properties.

**Surface Modification.** A variety of surface modifications have been undertaken in an attempt to create surfaces ideal for cell culture. Generally, these can be grouped into two types of modifications, biological and chemical. The biological surface
modifications generally involve deposition of biological macromolecules, such as fibronectin or collagen, on the surface to enhance cell attachment. Chemical modification can involve either chemical treatment of the surface or deposition of molecules on the surface. Usually, this is used to modify some physical parameter of the material such as surface energy.

The type of biological material deposited depends on the type of cell to be cultured and the desired effect of the modification. Not only can cell adhesion be selectively promoted, but substances such as bovine serum albumin (BSA) can prevent cell adhesion for some cell types. Often, both chemical and biological surface modification can be used in succession, with the surface modification promoting the adhesion of the biological molecule.

Two specific types of chemical modification of polydimethylsiloxane (PDMS), the elastomeric polymer used in this work, will be described later: surface treatment by hydrochloric acid immersion and electrostatic layer-by-layer (LbL) assembly of polyelectrolytes. The first method induces a charge on the surface, making it hydrophilic and suitable for adsorption of charged biological molecules. The layer-by-layer assembly alternates positively and negatively charged layers of polyelectrolytes to form a uniformly charged film. This is also useful for adsorbing biological molecules, and these may be included within the ultrathin films. Surface modification is used extensively in patterning cells on surfaces, as will be discussed later.
Current State of Muscle Tissue Engineering

Work resulting from studies on surface energy and toxicity of various materials towards cells has facilitated research in tissue engineering by providing insight into the selection of appropriate substrate components. Combined with advances in the production of very small features, a wide range of scaffolds has been produced for the purpose of controlling and measuring various properties of cell cultures. The various types of scaffolds currently in use in the field of tissue engineering, as specifically applied to muscle cells, the focus of this research, are summarized below.

Contact Guidance Scaffolds

Microgrooved Scaffolds. Contact guidance is the phenomenon of cell alignment defined by Dunn and Heath as “an oriented response of isolated tissue cells to the shape of the substratum such that the predominant direction of locomotion coincides with the direction of least curvature of the substratum.” This directional locomotion induces an alignment of cells along the axis of motion. A variety of structures can be designed to provoke contact guidance in tissue culture, given that they possess repetitive anisotropic features smaller than the size of the cell. Some of the more common scaffolds used are microscopic fibers and grooved surfaces.

One theory that has been explained for contact guidance on microscopic fibers, and may also be applicable for microgrooved surfaces, as shown in Figure 7, suggests that the actin filaments of an extending lamella cannot form when they would be bent. As a result, all points at which lamella form attachments must be points to which a cell can linearly extend an actin filament, precluding cells from forming such attachments around convex angles. Since it is necessary to make attachment plaques to a surface by
which to pull the main body of the cell forward, a cell cannot move in directions which are not conducive to forming these attachments. For microgrooved substrates, there is a probabilistic tendency for cells to make attachments in the directions of the grooves (rather than bridging across them), thereby traveling in those directions, and consequently aligning their long axes in that direction.\textsuperscript{53} This is further supported by data that show actin filaments are aligned in the direction of the grooves in cells grown on microgrooved substrates,\textsuperscript{55,56} and that the width and depth of the grooves are crucial to the degree of alignment produced.\textsuperscript{58}

Figure 7. Cells cultured on a microgrooved substrate

\textbf{Fiber Mesh Scaffolds.} One early type of structure designed for use as a tissue culture scaffold was a mesh matrix usually made of polymeric fibers\textsuperscript{59,60,61,62,63,64,65,66} such as polylactic acid (PLA), poly-L-lactic acid (PLLA), polyglycolic acid (PGA), or polylactic-co-glycolic acid (PLGA). Other materials, such as quartz fibers with diameters of 24 to 50 µm have been used to study the effects of cylindrical surfaces on cell attachment and morphology.\textsuperscript{67,68} Biological materials such as chitosan, which was used as a non-fibrous material for scaffold production,\textsuperscript{69} and collagen, a naturally-ordered fiber,\textsuperscript{70} have also been used. These polymers have an advantage over many others in that
they are degradable in the body, with a controlled resorption rate. Consequently, these
have been commonly used in an attempt to produce a porous three-dimensional mesh
scaffold that could be seeded with cells and implanted in the body. Another advantage to
the use of this type of scaffold is they can easily be produced in a variety of shapes,
allowing the scaffold to dictate the shape of the tissue. Alignment of cells can be
achieved by contact guidance as described earlier, with cells aligning with the axis of the
fiber as shown in Figure 8.

![Cells cultured on a fiber mesh scaffold](image)

**Figure 8. Cells cultured on a fiber mesh scaffold**

Despite these advantages, there are a number of problems facing the use of such
substrates. The culture of cells in a porous structure does not in itself constitute a
functional tissue. While some structural aspects of the tissue, such as cell density, may
be controllable, the tissue function is rarely replicated by these methods. That is not to
say, however, that these scaffolds are not useful. In instances where the tissue function is
not complex or is primarily structural, these show promise, such as in cosmetic
replacement of tissue or cartilage grafts.
One final observation about this type of tissue culture scaffold is that it is difficult to control with precision the microarchitecture of the substrate. While pore size, scaffold surface area to volume ratio, and other parameters can be controlled within a range, there is still some variation within the structure. Furthermore, microscopic features are difficult to design into the structure. As a result, scaffold geometry is highly limited by the fabrication process.

**Adhesive Micropatterns**

Cell micropatterning is an area that shows much promise for tissue engineering because of its ability to precisely control cell attachment spatially. The purpose behind cell patterning is to precisely control the size, shape, and location of cells grown in culture, allowing for detailed investigations of the effect of these parameters on the function of the tissue, or for use in cell based bioassay systems. It has been demonstrated that culturing cells on micropatterned adhesive islands promotes more aspects of normal cell function at an earlier point than on standard cell culture surfaces\(^{28}\). Although cell patterning is often used for applications other than tissue engineering, as mentioned in many of the instances below, its contribution to the field promises to be significant, allowing precise control of cells without the need for microstructural features.

**Photolithographic Micropatterns.** One method of patterning cells uses a standard photolithographic liftoff method.\(^{44,75,76}\) The deposition and subsequent exposure of photoresist is followed by deposition of one or more layers of protein or other molecules. The uncrosslinked photoresist is removed (with the overlying layers) leaving regions of two different degrees of adhesiveness: one attractive to cell attachment, and one that is cell-repellant as shown below in Figure 9. Another method of controlling cytophilicity
and cytophobicity, layer-by-layer (LbL) assembly of polyelectrolytes, is discussed later. Cell attachment can thus be confined to desired areas, as shown in Figure 10, though some cells may bridge across narrow cell-repellant areas as illustrated by Figure 11. Despite this, cell shape can be controlled by adhesive micropatterns with remarkable precision, especially on shapes without small, acute angles.\textsuperscript{75} A modification of this scheme involves developing the photoresist prior to deposition of the surface layers. The layers on the bare substrate can be preserved during the liftoff process.\textsuperscript{14} One problem that can be encountered is unsuccessful lift-off due to the layered film being too thick to be fractured during sonication.\textsuperscript{75}

![Cells cultured on adhesive photolithographic micropatterns](image9)

**Figure 9.** Cells cultured on adhesive photolithographic micropatterns

![Smooth muscle cells cultured on fibronectin micropatterns surrounded by PDDA cell repellant areas](image10)

**Figure 10.** Smooth muscle cells cultured on fibronectin micropatterns surrounded by PDDA cell repellant areas\textsuperscript{77}
A related method of producing patterned areas for cell adhesion involves photochemical micropatterning. Photochemical micropatterning alters the adhesion or structure of a deposited chemical by UV irradiation, or radio frequency glow discharge (RFGD). This allows fewer steps to complete the patterning process, but significantly restricts the number of available coatings for patterning the surface.

Microcontact Printing. Another technology, microcontact printing (µCP), involves the process of producing an elastomeric stamp with raised features that correspond to the patterned areas of the stamped substrate. The stamp is coated with a solution of “ink” (the material to be deposited), which is then stamped onto the desired substrate. As an example, this has been used to produce a substrate with patterns in hexadecanethiol on gold corresponding to the raised features on the stamp. The substrate is then immersed in another solution to prevent adhesion in the areas not contacted by the stamp. Next, proteins can be deposited onto the surface to form patterned adhesive areas to aid cell growth. Alternatively, proteins can be used as “ink” to directly pattern them by the same stamping procedure. An alternative uses the

Figure 11. Fluorescence micrograph of cells cultured on fibronectin micropatterns bridging across cell-repellant PDDA
stamp itself as a cell culture substrate, with the raised areas being coated with an adhesive protein and the recessed areas remaining nonadhesive.\textsuperscript{85}

Figure 12. Microcontact printing process (top) and cells cultured on microcontact printed substrates (bottom)

Microfluidic and Stencil Patterning. These two essentially equivalent techniques are used to convey a solution containing the adhesive to the cell culture substrate by elastomeric structures, which restrict contact with the solution to the nonadhesive areas by conformal contact. The stencils are simply silicone rubber membranes with holes corresponding to the areas intended to be patterned\textsuperscript{82} as shown in Figure 13. More complex patterns can be created by microfluidic patterning, which utilizes complex microfluidic networks to deliver solutions to the desired areas\textsuperscript{82,86,87,88,89} as shown in Figure 14. Patterning is not limited to creation of adhesive areas; cells can be patterned in this manner as well.\textsuperscript{87} The microfluidic network or stencil is left in place while the
cells attach, and then removed. However, cells will tend to migrate into the unpatterned areas given enough time. One difficulty with microfluidic patterning is that the flow pressures used are dependent on the structure geometry, which requires testing for each design to determine the optimal pressure.\textsuperscript{87}

Figure 13. Microstencil patterning (top) with cells cultured on patterned substrate (bottom)

Figure 14. Microfluidic patterning (top) and cells cultured on patterned substrate (bottom)
A modification of this method uses the microfluidic channels themselves to control the location of the cells permanently.\textsuperscript{86,87} Cells are seeded as in the microfluidic patterning, but the channels are left in place to guide cell patterning rather than an adhesive micropattern. The microfluidic network can simply be peeled off when desired if physical access to the cells is required. Otherwise, the channels can be left in place. One disadvantage of this method over the above adhesive micropatterning is that cell culture media must be continuously pumped into the device to prevent cell death, which requires a more complex setup. Also, adhesion of the membrane may be difficult to ensure over long culture periods or for very small channel wall dimensions. If the membrane is to be removed, an effort must be made to prevent cell adhesion to the membrane, which might cause damage to the underlying cells.

Other Micropatterning Methods. There are a variety of other methods used to produce patterns of adhesive and nonadhesive areas. One method produces honeycomb shaped patterns by preparing amphiphilic polymer solutions under high humidity, and this requires no lithography.\textsuperscript{90} This method has the drawback that it allows only limited control over the pattern architecture, providing minimal use to the biologist or biomedical engineer trying to design patterns to elicit a specific response.

Microchannel Scaffolds

Although surface chemistry is an important phenomenon that influences cell growth and behavior, and is useful for patterning cells on two dimensional surfaces, a three dimensional surface is needed for patterning of cells to form thick tissues. Like microfiber meshes, microchannel scaffolds offer a high surface area to volume ratio, which is essential for dense cultures. However, because of the manufacturing methods
used, much more complex topographies can be fabricated with a much higher geometric uniformity throughout the scaffold.

Microchannel and Micropillar Scaffolds. In order to elucidate the mechanisms in which scaffolds affect cell behavior, it is necessary to fabricate scaffolds and maintain a high degree of control over their design. As a result, what is needed is tissue culture scaffolds designed without limits placed on their microarchitecture by the chosen manufacturing method. Many scaffolds making use of various microfabrication processes have controlled feature sizes on the order of microns. One such structure was designed for the culture of hepatocytes with a high cell density. The scaffold consisted of an array of cubic wells in which cells were cultured. This provided a very controlled surface area-to-volume ratio. There are a variety of other scaffolds incorporating microchannels and micropillars, such as in Figure 15 and Figure 16, respectively. Here, microchannels are differentiated from microgrooves used in contact guidance studies by the width of the channel or groove. Channels equal to or greater than the average cell width are herein called microchannels, whereas those less than the cell width are called microgrooves. This distinction is made due to the different mechanisms of cell alignment. Microchannels align cells by forming physical barriers to cell movement in directions other than the desired axis, as in those used for microfluidic patterning of cells mentioned above. Microgrooves align cells by contact guidance as previously discussed and can be used in combination with microchannels. In general, cells grow in microchannels but on top of microgrooves. Micropillar is the term used here to refer to pillar structures that are designed to promote cell orientation by promoting
cell attachment to the side of the pillar. This differs from similar but smaller structures used in contact guidance studies where the cells grew on top of the pillars.

![Cells cultured in a microchannel scaffold](image1)

**Figure 15.** Cells cultured in a microchannel scaffold

![Cells cultured on a micropillar scaffold](image2)

**Figure 16.** Cells cultured on a micropillar scaffold

Microchannels and micropillars have some definite advantages over the previously mentioned mesh scaffolds. All the dimensions (depth, width, and height) can be precisely controlled, which is not easy with mesh scaffolds. Furthermore, the hollow areas of mesh scaffolds are essentially random, while those of microfabricated structures can be made uniform throughout.

There are disadvantages to the use of microchannels and micropillars for tissue culture as well. While it is relatively simple to design a single-level channeled device, the procedures for fabricating a multilayered scaffold are more complex. That is not to
say it is impossible, as structures have been fabricated with multiple layers of channels.\textsuperscript{98} For three-dimensional tissues that do not require complex organization, however, mesh scaffolds might be more economical.

Microchannels have been crucial in research devoted to engineering skin equivalents with structures similar to native skin. Channels have been fabricated for mimicking the basal lamina, with channel widths and depths varying from 40 to 200 µm.\textsuperscript{95} In this case, the purpose of the channels was to mimic the \textit{in vivo} structure of the underlying tissue, not to provide cellular alignment.

One example of the use of micropillars is a scaffold consisting of 5 µm high pegs with 10 µm diameters.\textsuperscript{30,92} Desai et al. found that cardiac myocytes tended to grow with one or more ends terminating at a peg.\textsuperscript{30} This shows that cells can be induced to orient themselves with an appropriately designed cell culture scaffold. Cells in this instance were not prevented from growing perpendicular to the intended direction, allowing a large number of cells to be misaligned. A modification of this scaffold placed the micropillars inside microchannels to provide cell alignment.\textsuperscript{99} It should also be noted that the ends of the cells were designed to attach to the scaffold itself, not other cells, potentially compromising the structural integrity of the tissue should it be fabricated from a resorbable material and implanted in the body. Furthermore, it limits the study to that of individual cells, not an entire tissue whose interconnections between cells are necessary for its proper function.

\textbf{Three-Dimensional Printing.} Another technique for producing tissue culture scaffolds is three-dimensional printing (3-DP).\textsuperscript{59} Scaffolds can be produced layer by layer, by printing a series of two dimensional structures in succession vertically.
Materials used include PLLA and chitosan, which are bioresorbable materials frequently used in tissue engineering scaffolds. Similarly, stereo lithography has been used to fabricate scaffolds.\textsuperscript{71} For research purposes, any scaffold that does not provide optical access to the cells growing in the structure has disadvantages due to the difficulty in obtaining data about the effect of the scaffold on the cells. As a result, it is often perhaps better to replicate a tissue in only two dimensions to provide adequate physical and optical access to cells for data acquisition, extending to three dimensions once the tissue properties are fully known. These techniques also suffer from being rather slow, and thus they are not suited for production of large numbers of substrates.

**Stress Scaffolds**

**Mechanical Deformation.** One method of controlling a cell’s phenotype relies on providing tensile stress to the cell.\textsuperscript{100,101,102} It has been shown that cells that receive this stimulus become more aligned,\textsuperscript{100,101} increase assembly of microtubules,\textsuperscript{102} and possess higher actin densities.\textsuperscript{100} Tension can be applied to cells by either deforming an entire tissue,\textsuperscript{100,101} or stretching a membrane on which cells are attached.\textsuperscript{30} It has been found that this latter method can cause cells to become detached from the underlying membrane.\textsuperscript{30}

There are several theories as to why cells realign in response to tensile stress. Use of *in vivo* tissue samples has shown that it is not simply a selective detachment of cells that causes the average alignment to be improved.\textsuperscript{100,101} Liu and Fung showed that tensile stress applied to smooth muscle tissue caused depolymerization of actin filaments in cells not aligned with the axis of stress, followed by cell death, and proliferation of SMCs that were aligned with the tensile stress axis.\textsuperscript{100} Two potential causes for the sudden
depolymerization are destruction of intercellular contacts and mechanical disruption of the actin filaments themselves.\textsuperscript{100}

**Fluid Shear Stress.** Another type of scaffold that has shown promise in aligning cells is scaffolds that use fluid shear stress.\textsuperscript{103} *In vivo* smooth muscle cells align circumferentially in arteries, perpendicular to the direction of fluid flow. For this reason, flowing media through scaffolds should cause alignment of smooth muscle cells as illustrated by Figure 17. The mechanism of alignment is different in fluid shear stress than in mechanical deformation, since it has been shown that in the latter, both SMCs and endothelial cells (ECs) align with the axis of stretch, whereas ECs align parallel and SMCs perpendicular in fluid flow scaffolds.\textsuperscript{103} Despite this, there is conflicting evidence with some studies showing little or no effect on alignment of SMCs by fluid flow.\textsuperscript{104,105} As a result, more research is needed to determine the mechanism by which fluid flow affects cellular orientation.

![Figure 17. Smooth muscle cells aligned in channels by flowing media](image)
Other Methods for Tissue Engineering

Another method of patterning cells actually involves the patterning of cells, rather than the patterning of substrates. Cells have been patterned by a system known as Matrix Assisted Pulsed Laser Evaporation Direct Write (MAPLE DW), which uses a pulsed laser to transfer cells to a desired substrate with a user controlled pattern. If cells could be consistently transferred at high viability rates, three-dimensional tissues could potentially be developed. However, simply patterning cells without a substrate that recreates the acellular components of tissue will not suffice. Eventually, it may be necessary to combine different methods of fabrication of substrates to take advantage of the various strengths of each.

While there are many types of cell culture scaffolds currently in use, they all have shortcomings. In general, those that offer high precision of cell placement are only two-dimensional scaffolds, while truly three-dimensional scaffolds do not usually support multiple cells types or allow for deposition of cells in a controlled fashion. Finally, while there are many intricate structural patterns that can be developed, the composition of the scaffold is at least as important in terms of how the cell interacts with the substrate. Since many fabrication methods determine the type of material from which the scaffold can be made, techniques that can alter the surface composition of the scaffold will be extremely helpful.

Application of Layer-by-Layer Assembly to Cell Culture

Layer-by-layer (LbL) assembly, as a method of coating solid substrates, has many promising features that make this technology extremely useful as a technique for
modifying the surface chemistry of cell culture substrates to promote cell adhesion, selectively pattern cells, and potentially control cell function.\textsuperscript{32,34,35,46,47,48} LbL typically involves the sequential deposition of positively and negatively charged polyelectrolytes, which are large charged water-soluble polymers. Nearly any surface can be layered with polyelectrolytes, given that it has sufficient charge to allow electrostatic attraction of polyions. Each successive layer is deposited and adsorbs to the surface by electrostatic attraction, with each layer possessing a sufficient excess of charge to cause a charge reversal with each new layer formed. Since the addition of a positive layer to a negative layer does not neutralize the surface, but instead presents a positive charge, this technique allows the deposition of an infinite number of layers. It also follows, since polyelectrolytes will not layer directly onto other polyelectrolytes with a like charge, that the thickness or uniformity of each layer is not highly dependent on time of immersion in the layering solution once a monolayer has formed.

Since the layers are held in place by electrostatic attraction, they form extremely stable layers on a variety of substrates. Unlike some thin film deposition processes, any surface geometry can be efficiently and uniformly coated, including tortuous microfluidic channels and surfaces with high aspect ratio structures. This makes LbL well suited for layering of cell culture substrates, which often must have highly complex surface geometries with features on the micron scale. Many other unique features of LbL make it advantageous for cell culture. Proteins, DNA, and even enzymes can be immobilized, either on a surface or within layers, while maintaining much of their functionality.\textsuperscript{35} Fluorescent layers can be formed by conjugating the polyelectrolytes to fluorophores, which is extremely useful in visualizing micropatterns of polyelectrolytes. By combining
LbL with traditional photolithographic techniques, patterning polyelectrolytes laterally with micron precision and feature size, substrates can be modified to present highly varied and highly controlled surface charges and chemistries to cells cultured on the surface. This allows for the production of cytophilic and cytophobic surfaces, which are useful for patterning cells. In fact, by varying the pH of the polyelectrolyte solution used during deposition, surface chemistries can be chosen from a continuous spectrum of cytophilic and cytophobic surfaces. With polymers selected from a large number of polyelectrolytes, the resulting surface can be individually tailored to the needs of the cell culture system.

Unlike many technologies which have a tradeoff between usefulness and complexity, LbL assembly is very simple to learn and apply. Since sequential dipping in solutions of polyelectrolytes is all that is necessary, there is very limited startup cost involved. Finally, since layer thicknesses are only nanometers thick and layering times are short, the entire process of surface modification by LbL assembly can be accomplished quickly for substrates layered with only a few layers. Although a few layers are all that is often required, hundreds of layers can be built up quickly due to the short process time required. In summary, LbL is an enabling technology that allows for precise definition of surface chemistries for cell culture substrates that are essential for full optimization of any tissue culture system.

**Research Objectives**

The goal of this research is to determine the effectiveness of microchannels for the culture of highly aligned, highly dense, contractile smooth muscle cells. The
existence of a causal relationship between smooth muscle cell alignment and phenotype will be investigated. Aspects of the cultured tissue to be analyzed are cell density, cell alignment, and cell phenotype. Alignment of cells will be measured by recording the acute angles between cells and the channel walls. Cell phenotype will be assessed by quantifying the amount of actin and myosin in smooth muscle cells. These data will be collected from cells grown in microchannels with varying channel widths to elucidate the relationship between channel width and the alignment, density, and phenotype of those cells.

**Hypotheses**

There are many factors that affect cell phenotype. For the purpose of determining the efficacy of the use of microchannels for regulating phenotypic expression, only one proposed factor, channel width, will be varied so as to ascertain its effect. Culturing cells in structures with interconnected microchannels will result in cell alignment. The channel widths will be chosen expressly to physically limit misalignment of cells. It is believed that by forcing cells to grow in narrow channels, they will exhibit shapes that are more elongated than those found on ordinary tissue culture substrates. This is important since it has been demonstrated that cellular function is dependent on cell shape. The cells will be aligned with the channels, allowing a cellular contraction to produce a contraction of the entire tissue along one axis should the scaffold be removed. It is believed that the cells will become more contractile as a result of a higher density of actin and myosin.

Forcing cells to grow in narrow channels will slow the spreading of the culture. It has been shown that forcing cells to cease proliferation earlier positively affects normal
cell function at an earlier state than if cell spreading is not controlled. The contractile cells should also be less dense due to the lower proliferation rates of these cells. Therefore, as the channel width is decreased, cells should become more highly aligned and exhibit more markers of a contractile phenotype. Cell density, a result of a high proliferation rate, should increase with the channel width.

It is believed that it is essential for the channels to be interconnected so that the resulting cells will form links with one another to produce a single tissue. Although this will result in an inherently less aligned tissue than if there were no interconnections, the breaks in the channel walls are essential so that the cells will form a single two-dimensional tissue of physically linked cells, rather than a series of isolated one-dimensional tissues that would necessarily form if cells could not connect between channels.

Outline of Thesis

This thesis is organized in the following manner: Chapter 2 covers the design criteria for the tissue culture scaffold, describing both the biological and structural factors considered in the design process. This chapter also describes the fabrication of the scaffold and the analysis methods used to evaluate the cell culture results. Chapter 3 presents the results obtained from the experiments and analysis of the data, with the fourth and final chapter discussing the findings from the data collected, and interpreting the analysis results. Appendices are included, listing MATLAB and SAS codes used in the analysis of the data.
CHAPTER 2

MATERIALS AND METHODS

Design of a Tissue Culture Scaffold

Biological Factors

Design of a tissue culture scaffold strongly depends upon a number of biological factors encountered in cell culture. These factors serve as constraints on the dimensions, materials, and geometric layout of the scaffold. One very important restriction is on the type of material from which the scaffold may be fabricated. The material chosen must be biocompatible so that it is not toxic to the cell. This toxicity is not limited in scope to whether or not the material can cause cell death, but whether the material causes a difference in the cell function and behavior as opposed to cells found in vivo. Another material consideration that has biological implications is the surface charge of the scaffold material. It is known that cell adhesion is greatly enhanced by the presence of a surface charge. Materials should be selected to promote cell adhesion if cell density is to be maximized.

The geometric design of the substrate is also vital to inducing cell behavior similar to that found in vivo. Muscle cells in vivo are highly aligned such that the long axes of the cells are parallel to one another. When the muscle cells contract in unison, the result is a contraction of the overall muscle tissue. However, in standard cell culture,
muscle cells grow in random directions, showing some self-induced alignment locally; nevertheless, it renders a contraction of the cells ineffectual in producing substantial useful mechanical deformation. Thus, an important characteristic of a cell culture scaffold is that it must induce the desired alignment of cells produced \textit{in vivo} by stimuli not usually present \textit{in vitro}. Oxygen and nutrient transport are essential to produce a healthy cell culture, and any cell culture scaffold must be designed to facilitate proper diffusion of cell culture media. In traditional monolayer cultures, transport is not an issue because every cell has access to the overlying media. If dense multilayer, tissue-like cultures are to be grown, the designer must take into account some method of providing sufficient transport to the entire cell culture.

The dimensions of the design of the structure are also dictated by the biology of the cell type to be cultured. Various dimensions to be considered are the dimensions of the cell when attached to the scaffold and the cell dimensions when in suspension, which may be a factor in cell seeding. The ability of a structure to induce alignment is dependent on its dimensions, which are, in turn, dependent on the cellular dimensions.

How the cells interact with one another in culture is also important. For contractile muscle cells to produce beneficial mechanical work, they must connect with one another for the purpose of transmission of contractile impulses. Also, if the cells are not appropriately connected, the cells will contract in isolation and not contract the overall tissue mass.

\textbf{Materials Factors}

A second, and often more challenging set of constraints, involves the properties of the scaffold materials themselves. Perhaps the most difficult problem to solve is finding
a manufacturing process to produce the desired cell culture scaffold. The choice of a material and a method of micromanufacturing usually go hand-in-hand, as one often dictates or severely limits the other. The structure should be designed to take advantage of all the abilities of a fabrication method and to minimize its deficiencies.

Probably the most important qualification of a micromanufacturing method in tissue engineering is that it is capable of producing small features (typically less than ten microns) on the same scale as the cells, and high aspect ratios—the ratio of the structure height to width. Aspect ratios of five to ten or more may be required to produce three-dimensional tissue scaffolds. When small features are required with high aspect ratios, one must also consider the strength of the material. It must be sufficient not only for the fabrication process, but also for the intended use of the device.

There are a variety of other requirements for the scaffold material that are application-specific. One is the optical transparency of the material. For tissue culture scaffolds that must be observed under a microscope, it is crucial to have optically transparent structures and be thin enough to be viewable with high magnification objectives. It is also important to choose a material that can be readily sterilized. Some materials cannot be subjected to heat sterilization; others may dissolve in alcohol or break down when irradiated by UV light.

**Scaffold Design**

The above criteria were considered during an iterative process of scaffold design. The first scaffold designed consisted of blind vertical channels intended to be more than 100 µm deep to allow cells to be able to fully elongate. Four types of channel geometries were designed: square channels, octagonal channels to investigate the effects of obtuse
angles, triangular channels to investigate the effects of acute angles, and plus shaped channels. The last design was intended to provide a diffusion channel by which to supply nutrients to the cells growing inside the scaffold, surrounded by four channels to grow cells. The triangular design had to be eliminated due to limitations on exposure geometry. All the channel areas on the mask needed to be exposed by a rectangular window, eliminating channels with acute angles.

The manufacturing process chosen to quickly produce large numbers of scaffolds was PDMS molding. Posts of up to 50 µm were formed that were negative structures of the channels to be molded by photolithography using a negative photoresist patterned on a silicon wafer by standard photolithographic techniques. It was found, however, that the scaffold made of PDMS could not be released from the wafer without tearing. Therefore, it was necessary to design a new mask that could be effectively used to produce PDMS scaffolds.

The next scaffold was to consist of three types of structures to be used independently. The least complex were long interconnected channels exposed along their entire length to the cell culture media. A second type of structure, a modification of this design, included diffusion channels to allow for media flow. Wells were designed to provide a media inlet and outlet. With this design, it would be possible to produce multilayered scaffolds, fulfilling the desire of a fully three-dimensional scaffold to produce aligned muscle cells. Since the effect of the channels on the tissue can be more easily tested on a two dimensional scaffold due to the measurement difficulties of three dimensional systems, this was not actively pursued. A final section of the scaffold consisted of channels aligned circumferentially to produce muscle tissue capable of
constricting a vessel, demonstrating the capabilities of microfabricated cell culture substrates not found in mesh scaffolds. Once again, the difficulty in producing the mask from the design resulted in the elimination of this culture area from the final design.

With the last two types of scaffold patterns eliminated as described, the remaining portion (the first type of scaffold listed) was very useful in examining the effect of channels on cell phenotype. The purpose of the channels is to impose alignment on the cells by physical barriers to migration and orientation. A basic requirement of the scaffold is to allow for maximal cell-to-cell interaction. Cells must be able to form multiple contacts with other cells for a viable tissue to be formed. Therefore, sizable openings (50 µm) were left in the channel walls to allow cross-channel interaction. Without these openings in the channel walls, the cells could grow in long strings of cells, but could not be a functional sheet of tissue.

Despite the relative simplicity of the design, there were several critical decisions to be made. The width of the channels was the most important factor. Due to the expense of the exposure time and the necessity of having sufficient culture area for each section of the scaffold, the number of different channel widths that could be investigated was limited. For this reason, channel widths greater than 60 µm were not designed because the size of the cells in relation to the channels was too small to expect this to be the optimal width. A minimum width was set at 20 µm because it was felt that while channels allowing only one row of cells to attach would be possible with smaller dimensions, cells grown in isolation were not likely to provide data applicable to cell phenotype of SMCs in *in vivo* tissue. That is, the communal effect of the cells on each other must be incorporated in the design, and a scaffold consisting of single rows of cells
in isolation did not appear to be a valid design. A channel of 20 µm would allow sufficient room for all cells to interact with each other and still be able to contact the channel walls.

The channel depth was another important dimension that needed to be decided at the mask design stage. Although channel depth is not actually controlled by the mask itself, the limitations placed on structure stability during mold release require that the decision be made at this point. The governing factor in the demolding process is the aspect ratio, or ratio of height to width of a feature. Channel walls with high aspect ratios will be difficult to remove from the mold without tearing. Shearing of PDMS channel walls inside the silicon wafer channels, as shown in Figure 18, effectively prevents further use of the Si wafer for making more silicone substrates. Because channel wall height was more important physiologically than channel wall width, or thickness, the depth of the channels was selected first.

![Figure 18. Scanning electron microscope image of PDMS fragment sheared from scaffold during mold release](image)

Several factors influence the decision on channel depth. The channel must not be so deep that it begins to cause nutrient deprivation at the bottom of the channels. If it did,
this would more adversely affect cells in the narrow channels than it would those in the wider channels. A maximum ratio of height to width was selected as 1:1 since a channel with height exceeding the width would be equivalent of a channel with interchanged height and width. With the minimum channel width set at 20 µm, this resulted in the selection of 20 micron deep channels, which are deep enough to prevent cells from growing over the walls, but not so deep that cells could grow with a significant vertical component to their orientations. This depth was later changed to approximately 10 µm to facilitate demolding. Next, the width of the channel walls needed to be selected. As the channel walls are made thicker, the stronger they become. This tends to significantly limit cell growth area as well. Thus, two channel wall widths were selected, one conservative (10 µm), and one more optimistic (5 µm). The aspect ratios of the two are one and two, respectively.

Manufacture of a Tissue Culture Scaffold

Mask Production

First, the mask design was drawn using AutoCAD 14 software (Autodesk) on a personal computer. The entire design consists of closed rectangles that correspond to the transparent areas of the mask. One set of structures consists of parallel, interconnected channels with intervening diffusion channels for media flow, as shown in Figure 19. There are two variations on this design that have different channel widths. Another set of structures consists of parallel, interconnected channels without diffusion channels illustrated in Figure 20. Once the design was finalized, it was sent to LSI Photomask which produced a 5 in by 5 in chrome on glass optical mask.
Figure 19. Tissue culture scaffold with interconnected channels and diffusion channels

Figure 20. Tissue culture scaffold design with interconnected channels
ICP Etch of Silicon Wafer

A positive photoresist (AZ-1813) was spun on a silicon wafer to a thickness of 1.6 µm. The mask was used to pattern the photoresist by exposing it to UV light using standard lithographic techniques. The wafer was then dry-etched with ICP, as shown in Figure 21, to produce the negative structure of the scaffolds desired, in silicon, to a depth of approximately 10 µm. The wafer was then rinsed in acetone to remove the residual photoresist, rinsed in isopropyl alcohol and deionized (DI) water, then dried and observed using an SEM (scanning electron microscope). Photographs are presented later in the section concerning metrology. A flow chart of the silicon wafer production process is shown below in Figure 22.

![Diagram of Inductively Coupled Plasma (ICP) etch process](image)

Figure 21. Diagram of Inductively Coupled Plasma (ICP) etch process
Production of Microscaffolds

First, the wafer was rinsed with 99% isopropanol, then deionized water to clean, and dried with nitrogen gas. The scaffolds used for cell culture were produced by PDMS molding with only slight modifications to a previous protocol. Sylgard 184 Polydimethylsiloxane (Dow Corning) was purchased in a kit containing silicone elastomer and a curing agent. The two components were mixed in a 10:1 weight ratio of elastomer to curing agent. The silicone mixture was poured over the surface of the silicon wafer with the microstructures to be replicated. The wafer was then placed in a vacuum chamber to remove air bubbles formed by mixing as well as those trapped within the etched features. To speed the curing process, the wafer was then placed in an oven.
for 1.5 hours at 70°C. After the silicone had fully cured, it was allowed to cool before being peeled from the silicon wafer. It is essential to peel the silicone in the direction of the channels to prevent tearing of the structures.

**Surface Modification of Substrates**

**Substrate Cleaning.** The scaffolds must be cleaned to remove any particles or oil that has adhered to the scaffold during the production process. The cleaning solution used was a 1% solution of citranox detergent in deionized water. Scaffolds were first sonicated in an ultrasonic bath in the detergent solution for ten minutes to loosen dust and other particulates trapped on the surface of the PDMS. Next, the substrates were given a vacuum rinse in a detergent as follows. The cell culture substrates were placed in fresh detergent solution and placed in a vacuum chamber. They were subsequently subjected to very low pressure (>29 in Hg vacuum) in the vacuum chamber to draw out air bubbles that were formed in the channels of the structure when the liquid was poured over the surface. The air was then allowed to reenter the vacuum chamber, and the scaffolds remained in the solution for 30 minutes.

A 75% solution of ethanol in water was used for a second vacuum rinse. The cell culture substrates were placed in the vacuum chamber as previously described and submerged in the ethanol solution. They were vacuumed until all air bubbles were removed and then subjected to atmospheric pressure for 30 minutes. The structures were then rinsed in ultrapure 18 MΩ-cm Millipore (Type I) water and submerged for temporary storage to prevent oil, dust, and other particles from contaminating the surface.
HCl Treatment. The PDMS substrates are naturally hydrophobic, making it difficult for cells or ECM proteins to adhere to the surface. One method of modification of the PDMS surface that was used involves treatment with hydrochloric acid.\textsuperscript{30} The hydrophobic silicone substrates were submerged in 10 N HCl for 2 hours to render them hydrophilic. Upon removal from the acid, the structures were hydrophilic.

There are a few drawbacks to this method of surface modification. It has been reported that the water contact angle on oxidized PDMS changes from 30° to its original value in 45 minutes after exposure to air.\textsuperscript{109} The recommendation to keep the substrates submerged in water upon treatment whether by plasma oxidation or HCl is often not practical. Treatment of the PDMS substrates with HCl has also caused curling of the membranes, perhaps due to surface stresses, making it more difficult to ensure the substrates stay submerged during cell culture.

The most challenging problem to overcome would be the lack of transparency the treated PDMS possesses. The substrates must remain optically transparent to permit microscopic examination of cells cultured on the surface of the substrate. For this reason, HCl surface modification, to create a hydrophilic surface for cell and protein attachment that can be observed by microscope, is not feasible.

Layer-by-Layer Assembly. Another method to create a charged surface on the PDMS is accomplished by a polyelectrolyte layering technique.\textsuperscript{32,34} Charged polymers were layered on a surface in alternating layers of positive and negative polymers. The ionic attraction suffices to bind the polymer layers. Four different polyelectrolytes were used to perform layer-by-layer assembly on the PDMS substrates. The positively charged polymers, polyethyleneimine (PEI), poly(diallyldimethylammonium chloride)
(PDDA) (molecular weight, 100 kDa – 200 kDa), and poly-l-lysine (PLL) (molecular weight, 70 kDa – 150 kDa) were obtained from Sigma. The negatively charged polyion, poly(sodium 4-styrene sulfonate) (PSS) (molecular weight, 1MDa), was also purchased from Sigma. Tris buffer with a pH of 7.6 was filtered through a 0.45 µm membrane prior to use in dissolving the polyelectrolytes as follows: PEI, 3 mg/mL, PSS, 2 mg/mL, and PDDA, 2 mg/mL. Type I water was used to dissolve PLL which was mixed at a concentration of 100 µg/mL and subsequently filtered through a 0.22 µm membrane to sterilize. All solutions and substrates were kept covered when not inside a laminar flow hood to prevent accumulation of dust within the solutions or on the cell culture scaffolds.

The PDMS was first submerged in the PEI solution for 30 minutes using a vacuum to remove air bubbles from the channels to ensure uniform deposition. After layering with PEI, the PDMS was rinsed with Tris buffer to remove the PEI, and vacuumed again to remove air bubbles. This process was repeated with PSS for 20 minutes followed by another rinse in Tris buffer. PDDA was then layered for 20 minutes and rinsed to produce one bilayer of PSS and PDDA. The layer-by-layer assembly process was continued until a polyelectrolyte multilayer film of the architecture PEI/(PSS/PDDA)₂/PSS is achieved. The substrates were then rinsed with DI water and dried until ready for use.

It is known that to reduce the IST of most materials, it is beneficial, if not necessary, to adsorb proteins or other substances to their surface that induce attachment. PLL was chosen because it has been used previously in preparing cell adhesive layers, and because it can be electrostatically layered due to its positive charge. PLL
hydrobromide (Figure 23) of molecular weight 70–150 kDa was obtained for the purpose of layering the cell culture scaffolds to produce a surface that induces cell attachment.

\[
\left( \text{H}_2\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}^+ \right)_n \cdot \text{HBr}
\]

Figure 23. Molecular structure of poly l-lysine hydrobromide (PLL)

Because the surface must be sterile, the scaffolds were sterilized by immersion in 75% ethanol (EtOH) for 30 minutes, using a vacuum to remove air bubbles from the channels as needed. The alcohol sterilization appears not to have any effect on the stability of the polyelectrolyte multilayer film.\(^{107}\) The alcohol was aspirated and the scaffolds dried by air flow inside a laminar flow hood. The structures were submerged in the PLL solution for 30 minutes following vacuum removal of air bubbles. The solution was removed, and the structures were dried under a sterile laminar flow hood overnight. Residual PLL was removed by rinsing in sterile ultrapure water prior to cell seeding. The process of layering the molding and layering the PDMS substrates is schematically shown in Figure 24.
Silicon wafer

Cure PDMS elastomer on wafer

Peel off PDMS substrate

Layer PDMS with polyelectrolytes

Figure 24. Production and layering of PDMS substrates

**Scaffold Metrology**

**Microchannel Structure Analysis**

A variety of methods were used to measure the width and depths of the microchannel structures. A roughness step tester (RST) was used to measure the depths and widths of the channels. The effectiveness of the method is highly dependent on the optical properties of the substrate to be analyzed. A surface profiler was used to measure the width and depths of the channels as well. This method employs a stylus that is moved across the surface of the substrate, recording the height. For narrow channels, the stylus may be too large to be inserted into the channels, resulting in incorrect measurements. Also, delicate structures, such as high aspect ratio channels made of PDMS, may deform under the pressure of the stylus, resulting in faulty data as well.

Another method of measuring the height and depth of PDMS channels utilizes images taken using an SEM. First, the PDMS substrate was sliced perpendicular to the
channel axis to provide cross sections of each set of channels. These were then sputter coated with palladium to facilitate imaging with the SEM. Pictures were taken of each channel cross section and were analyzed to determine the depth and widths of the microchannels. Unlike the RST and surface profiler methods, this technique is neither affected by the optical properties or the flexibility of the substrate.

**PLL Adhesion Analysis**

The FITC conjugated PLL was prepared as follows, based on an amine labeling technique from Molecular Probes, and designed to label approximately 1% of the available lysine monomers. First, 5 mg of PLL (Mw 70 kDa – 150 kDa) was dissolved in 1 mL of a 0.1 M sodium bicarbonate solution at pH 9. A stock solution of FITC was produced by dissolving 1 mg in 100 µL of DMF (dimethylformamide). The PLL mixture was then stirred for 3 hours upon addition of 10 µL of the FITC stock solution to fully react the two components. Acetone was added to precipitate the FITC labeled PLL out of solution. The acetone solution was centrifuged 4 minutes at low speed, and the acetone, with unreacted FITC still dissolved, was pipetted off. The PLL-FITC was resuspended in 50 mL of DI water to layer the PDMS substrate.

One PDMS substrate containing a full set of all the different microscaffolds used was layered as before, except the PLL layer was replaced with the FITC labeled PLL. Care was taken to avoid exposing the PLL-FITC solution or the layered structure to light to avoid photobleaching. A PDMS substrate layered with PLL and a glass slide (Daigger) layered with PLL-FITC were used as controls to eliminate the effect of autofluorescence from the PLL, PDMS, and polystyrene petri dish. Measurement was
performed on a Nikon TS-100F fluorescence microscope, using a standard filter cube designed for FITC. A Nikon Coolpix 996 digital camera was used to acquire images of the fluorescence. The exposure time was manually set to 2 seconds, with this and all other parameters, such as zoom, objective (40x), neutral density filter setting, etc., kept constant. A MATLAB (The MathWorks, Inc.) script (Appendix A) was written to calculate the mean of the green component of the image for each structure. Only small portions of each image were used to avoid analysis of the sides of the channel walls, which would be noticeably brighter.

Cell Culture Methods

Standard Cell Culture Technique

A variety of cell culture techniques are described here that were utilized in the undertaken research. RPMI 1640 cell culture media (HyClone) was used to culture all cells during experiments. It was mixed from a powder according to the recommended protocol, filtered with negative pressure through a 0.2 µm pore bottle top filter to sterilize, and supplemented with 1% sterile 100x antibiotic/antimycotic (AB/AM) from Sigma and 10% sterile fetal bovine serum (FBS), Lot G0031 from Atlanta Biologicals.

Sprague-Dawley rat aortic smooth muscle cells (RASMC) of passage number 2 were graciously donated by Dr. Kevin J. McCarthy at the Louisiana State University Health Sciences Center in Shreveport, Louisiana (LSUHSC-Shreveport). The cells were obtained frozen, suspended in cell freeze media, and stored in a liquid nitrogen tank until used. One vial of RASMCs was removed and the contents placed in a 15 mL tube and brought to 5 mL by addition of HBSS (Hanks’ Balanced Salt Solution) under a laminar
flow hood. The suspension was resuspended and placed in a tissue culture flask filled with sufficient media to cover the bottom surface of the flask. Because cells are extremely fragile after being thawed in the DMSO freeze media, they were not centrifuged to remove the freeze media, which might further damage the cells. Instead, the cell suspension was diluted with media, as mentioned above, to lessen the concentration of the residual DMSO. The flask was then placed in an incubator set at 37°C with a humidified atmosphere containing 5% CO₂.

Cells were observed under microscope on alternating days, prior to changing the media. Cells were passaged as recommended at subconfluency to ensure phenotypic expression was not altered by a loss of contact inhibition.10 Preceding passage, cells were twice rinsed in HBSS to remove the cell culture media from the flask. The HBSS was removed and trypsin (1x), formed by a 1:10 dilution of 10x trypsin in HBSS, was added to the flask which was incubated for 5 minutes at 37 °C. After the incubation period, the trypsin was agitated by a pipette to facilitate cell detachment from the flask. If necessary, a second incubation of 5 minutes was performed to remove the cells, but no more to prevent unnecessary cell stress. The cell suspension in trypsin was removed from the flask and placed in a sterile 15 mL tube and centrifuged for 5 minutes at 1000 rpm (200g) at 5 °C. The supernatant was removed and replaced with RPMI media, and the cells were resuspended. These cells could either be replated on tissue culture flasks to increase their number, or used for cell seeding into microscaffolds. Cells of passage 7 were used for seeding into the microchannels.

Before seeding cells into the scaffolds, it is necessary to quantify the cell density, as cell density at a given time after seeding until confluency is strongly dependent on the
initial seeding density. The technique used here, trypan blue exclusion, employs trypan blue (Sigma), a dye that is taken up by dead cells, but excluded by living cells. To employ this method, 200 µL of a very well mixed cell suspension, obtained by trypsinizing a tissue culture flask as described above, was placed in a 15 mL tube. To this was added 20 µL of 0.4% trypan blue and 160 µL HBSS. The cells were resuspended to prevent cells from clustering, thereby skewing the cell count. A coverslip was placed on the hemacytometer, under which a drop of cell suspension is placed. Living cells were counted and multiplied by 10,000 to obtain an estimate of the number of cells per mL of suspension. This test was repeated several times to ensure accuracy.

Good sterile technique is crucial to all cell culture work. All reagents and other materials, such as glassware, that were used with cells were obtained and kept sterile. Glassware, such as media bottles, was sterilized with dry heat in an autoclave for 15 minutes at 120°C. Pipette tips were also sterilized in this manner. Most other plastic cell culture supplies were obtained sterile. Sterile technique was practiced throughout, except in situations when nonsterile reagents could be used and the cells had already been fixed for staining, meaning contamination was no longer a problem. Prior to cell feeding, the air flow on a laminar flow hood was turned on, as was the UV light briefly, and all surfaces were wiped down with 75% ethanol in DI water. Once the ethanol had evaporated, the hood was ready for use. Care was taken to avoid contamination of reagents either with bacteria or each other, as described previously. Latex gloves were worn at all times, and care was taken to avoid placing nonsterile, or potentially nonsterile objects in the sterile air flow directly upwind from sterile reagents or cell cultures. The incubator was kept clean by weekly cleaning and sterilizing to prevent outbreaks of
microbes that might spread to the cell cultures. With adequate sterilization procedures in place, it was possible to prevent contamination of the cultures and reagents.

**In Vivo Control Studies**

A series of *in vivo* control studies was performed to provide a basis for comparison for the *in vitro* studies. Ideally, all of the quantities measured in the *in vitro* studies would be replicated in the *in vivo* studies. However, thin tissue samples of less than 20 µm proved difficult to obtain and of varying quality. In general, with the exception of the Hoechst 33342 micrographs, the *in vivo* tissue samples were too thick to provide analyzable images, even using a confocal microscope. However, they did demonstrate qualitatively that there was an abundance of contractile filaments present in the tissue. Some quantitative data was gathered in the nuclear alignment study which clearly demonstrates the alignment of SMCs *in vivo*. As a result, the primary purpose of the *in vivo* control studies was to provide qualitative demonstrations of the ability to detect the presence of actin and myosin, which are essential for the function of contractile smooth muscle cells.

**Tissue Sample Procurement.** All of the tissue samples were obtained in accordance with guidelines provided by the appropriate regulatory agencies, as described below. Five month old Sprague-Dawley rats were selected as controls for the experiment because the cells used in the *in vitro* studies were also from Sprague-Dawley rats, which are commonly used in biological experiments. The rats were housed at the Animal Care facility at Louisiana Tech University in a standard controlled environment as dictated by the Public Health Service Policy on Human Care and Use of Laboratory Animals, administered by the Institutional Animal Care and Use Committee.
As animals were needed for tissue samples, they were sacrificed by guillotine. The target tissues for the dissection were the descending aorta and the gut tubes, both of which possess sizable quantities of smooth muscle cells. The guidelines provided by the Department of Environmental Safety at Louisiana Tech University were followed in disposal of the remainder of the animal. The tissue samples were each sliced into eight sections to be fixed and stained.

**Tissue Sample Preparation.** The method of fixation is dependent on the type of stain to be used, as described in detail later. Two samples were chosen for nuclear staining and were fixed in 100% methanol for 20 minutes. To detect the presence of smooth muscle myosin, antibody staining was to be performed on tissue fixed in Methacarn solution for 2 hours. The remaining four samples were fixed in a 4% formaldehyde solution for 30 minutes. Two of these were to be stained with the actin stain, the other two with H&E (hematoxylin and eosin). Tissue dehydration was accomplished by two hour incubations in serial immersions of 75%, 90%, 95%, and 100% ethanol. This was followed by a four hour immersion in Histoclear™.

Samples were embedded in paraffin wax, and then secured by metal cassettes to prepare for microtoming. Each tissue was embedded so as to provide both cross-sectional and longitudinal cuts on the same slide for easy comparison. The embedded tissues were microtomed at 12 µm thickness and mounted on microscope slides previously treated by chrome alum. Images of the tissue samples were acquired as described later.
In Vitro Studies

Seeding of Cells into Scaffolds. A cell suspension was prepared from cells grown on tissue culture flasks as previously described, from which 200 µL was used for determination of cell density. The remaining suspension was diluted by adding RPMI. The structures layered with PLL were placed in sterile petri dishes. Sections of PDMS were cut so as to form a well encompassing the two rows of structures on each scaffold. The areas at the bottom of the two wells were calculated to be 2.8 cm² for the first row (structures I – VI), and 2.45 cm² for the second row (structures VII – X). It was determined through preliminary studies on different cell seeding densities that an optimal cell seeding density for use with a one-week culture is about 10,000 cells/cm². Given that the larger well can hold 700 µL of media, and the smaller well can hold about 610 µL, based on the surface areas of the two wells, cell suspension densities of 40,000 cells/mL must be used. The cells were applied, being resuspended immediately before seeding, and observed under light microscope. If air bubbles were lodged in the cell culture channels, the structures were vacuumed in a sterile vacuum chamber to remove the air bubbles. Care was taken to not vacuum excessively, potentially depriving the cells of oxygen for long periods of time. The cells were incubated for 6 days to allow sufficient attachment and spreading without such proliferation as would make quantification of cell density problematic. Cells were fed at two and four days after seeding and incubated as described earlier.

Staining Protocols

A variety of staining protocols are used to provide quantitative information about cellular qualities, such as cellular orientation, density, and contractile protein content.
The protocols described are standard techniques, and have been adapted to work with the culture system being tested.

Nucleic Acid Staining. Measurement of cell density was performed following staining by classical fluorescent nucleic acid stains. These stains target the nuclei, allowing for simple estimation of cell density from fluorescence micrographs of the stained cells. Hoechst 33342 (Molecular Probes), propidium iodide (Sigma), and ethidium homodimer-1 (Molecular Probes) were used to stain the nucleic acids of the cells. The molecular structures of these dyes are shown in Figure 25—Figure 27. Hoechst 33342 is a lipophilic dye that passes through the plasma membrane of cells, staining live and dead cells indiscriminately. The cell-permeant, minor groove-binding, Hoechst 33342 exhibits an increase in fluorescence upon binding to DNA. Because Hoechst 33342 binds to A-T regions with a greater frequency than at other base pairs, it is extremely selective for DNA over RNA. Both propidium iodide and ethidium homodimer-1 are intercalating cell-impermeant hydrophilic nucleic acid dyes. As a result, they only stain cells with leaky membranes, a hallmark of a nonviable cell. The differential staining patterns of the cell-permeant and cell-impermeant dyes allow use of a simple method for automated cell density and viability measurements, as described later. These dyes were tested in addition to trypan blue which, was not used due to the increased difficulty of automatically digitally isolating stained nuclei in bright field images. Manual counting does not improve the situation considerably, as it has been found that determination of viability of trypan blue-stained cells is often arbitrary and therefore not reliable. Also, it has been reported that trypan blue may overestimate cell viability when measurements are not taken immediately following staining. The
fluorescence spectra of Hoechst 33258, a dye with properties similar to Hoechst 33342,
and ethidium homodimer-1 when bound to DNA, are shown in Figure 28, demonstrating
their usefulness for simultaneous staining.

Figure 25. Molecular structure of Hoechst 33342 nucleic acid dye

Figure 26. Molecular structure of ethidium homodimer-1 nucleic acid dye

Figure 27. Molecular structure of propidium iodide nucleic acid dye
Both propidium iodide and ethidium homodimer-1 showed considerable cytoplasmic staining because of their ability to bind to both DNA and RNA. As a result, it was necessary to first treat the cells with RNase A, an enzyme that hydrolyzes RNA, reducing the overall staining of RNA by the nucleic acid dyes. It was found that ethidium homodimer-1 provided better contrast between nuclear and cytoplasmic staining following RNase A treatment than propidium iodide, as shown in Figure 29a and b, and was therefore used for all experiments.
Figure 29. (a) Smooth muscle cells stained with propidium iodide following treatment with RNase A show higher background with cytoplasmic staining than (b) cells stained with ethidium homodimer-1 following the same treatment with RNase A.

Stock solutions were prepared as follows. RNase A was prepared as a 100 µg/mL solution in phosphate buffered saline (PBS) and frozen in aliquots until needed. The Hoechst 33342 stock solution was prepared by diluting 10 mg/mL in RO (reverse osmosis) water and was refrigerated at 4°C until use. The ethidium homodimer-1 was obtained as a 2 mM solution in a 1:4 (v/v) ratio of dimethylsulfoxide (DMSO) and H₂O and stored in working aliquots for future use.

To stain the nuclei of adherent cells for fluorescence imaging, cells were first rinsed (three quick rinses followed by two five-minute rinses) in warm PBS. Next, cells were incubated for 20 minutes at 37°C in the solution of RNase A prepared above. The RNase A solution was removed by rinsing with PBS as described above. Both nucleic acid dyes were combined into one staining solution containing a 17.8 µM concentration of Hoechst 33342 and a 1 µM concentration of ethidium homodimer-1 in PBS. Cells were incubated in this solution for 40 minutes at 37°C and were rinsed in PBS immediately following.
The *in vivo* tissue samples were prepared as follows. First, the aorta was cut open and immobilized with surgical pins on a 1 cm² section of cardboard. The sample was fixed in 100% methanol for 24 hours. The sample was then placed in ethanol and can be stored for up to three weeks within the tissue cassette. To begin staining, the sample was submerged in PBS for two hours to remove the EtOH. The sample was removed from the cardboard and placed in a 24-well plate. The working solution of Hoechst 33342 was diluted from the stock solution as above and incubated for 20 minutes in the dark at room temperature. The sample was rinsed with PBS and submerged for one minute to remove excess stain not bound within the cells. Optionally, the tissue could be submerged in a solution of 50% glycerol in PBS for one hour to clear the sample. Finally, the tissue was submerged in 100% glycerol for one hour and may be stored in this manner for up to 72 hours prior to microscopy.

Cells were viewed with a fluorescence microscope using filter sets appropriate for DAPI (for Hoechst 33342) and TRITC (for ethidium homodimer-1). Because the cells do not remain viable for long periods of time after removing them from the cell culture media, it is necessary to observe the adherent cells soon after staining. Otherwise, the cells that initially stained only with Hoechst 33342 will also begin to be stained by ethidium homodimer-1, as it is not irreversibly bound to the nucleic acids. The nucleic acid staining described above is very useful, as it is utilized in cell density, viability, alignment, and elongation measurements.

**Filamentous Actin Staining.** Phalloidin conjugated with either of two different fluorophores was tested to stain the F-actin present in the cell cultures. Phalloidin, a toxin isolated from the mushroom *Amanita phalloides*, is extremely useful for aiding
visualization of actin filament structures because it binds specifically to actin. It also prevents actin depolymerization by binding between adjacent F-actin subunits.\textsuperscript{19} This results in a highly stable actin-phalloidin complex that, when the phalloidin is conjugated with a fluorophore, can yield high contrast images by fluorescence microscopy.

Fluorescein conjugated phalloidin (Molecular Probes) was first used to stain the actin filaments; however, difficulties with photobleaching required substitution with Alexa Fluor 488-phalloidin, also obtained from Molecular Probes. Both probes have nearly identical maximum excitation and emission wavelengths (496/516 for fluorescein versus 495/518 for Alexa Fluor 488, in nm),\textsuperscript{113} and they can be visualized with a standard FITC filter set. Alexa Fluor 488-phalloidin was dissolved in methanol to form a concentration of 200 units/mL, resulting in a 6.6 µM stock solution that could be frozen in aliquots for future use. Cells were rinsed with warmed PBS as above to completely remove the cell culture media. Following rinsing, cells were fixed for ten minutes at room temperature in a 4% solution of formaldehyde in PBS from paraformaldehyde produced as follows. A 2x solution of PBS was produced by dissolving 7.7 g NaOH and 33.6 g NaH$_2$PO$_4$ in 1 L DI water. An aliquot of 250 mL of the concentrated PBS solution was heated to 60°C, whereupon 20 g of granular paraformaldehyde was introduced and stirred until completely dissolved. After the addition of 250 mL of DI water, the solution was filtered and placed on ice to cool. For extended storage (up to one month), the formaldehyde solution was refrigerated.

After fixation, the cells were rinsed with PBS to remove the fixative and permeabilized with a 0.1% solution of Triton X-100 to allow entry of the hydrophilic phalloidin into the cells. The cells were incubated at room temperature for three to five
minutes and were rinsed with PBS prior to staining. The phalloidin stock solution was made by preparing a 1:40 dilution of the methanolic stock solution in PBS, resulting in a phalloidin concentration of 165 nM. This solution was applied for 20 minutes at room temperature to the cells and was removed by rinsing with PBS.

**Smooth Muscle Myosin Staining.** Cells were stained for smooth muscle myosin by an immunohistochemical procedure that is readily viewable with bright field microscopy. First, the cells to be stained were rinsed with warmed PBS, three times quickly, followed by two 5-minute rinses. Next, the cells were fixed with 4% formaldehyde for 10 minutes at room temperature to preserve the structure of the cell. The cells were then rinsed as before, but with a 0.05% solution of Tween 20 in PBS. A 1% solution of Triton X-100 in PBS was then applied for 10 minutes at room temperature to permeabilize the cells, allowing the antibodies to reach the interior of the cells and attach to the myosin. The Triton X-100 was then rinsed as before with PBS-Tween, which was substituted for PBS to preserve the cell permeability throughout the staining procedure. Next, a peroxide block was applied for 30 minutes at room temperature to remove any endogenous peroxidases that might interfere with the staining and produce a high background. The block was removed by PBS-Tween rinses and a protein block was applied for 30 minutes at room temperature. The staining procedure utilized reagents from an ABC Vectastain kit (Vector Laboratories), and many of the components, such as the protein block, were obtained from the kit and used as directed. When the protein block was removed, an IgG₁ antibody directed against smooth muscle myosin, clone hSM-V (Sigma) produced in mice, was applied without rinsing the cells. A dilution of 1:100 of antibody solution to antibody diluent (Dako) was prepared and applied for one
hour at 37°C, while the control sample was rinsed in PBS. The antibody was removed by PBS-Tween rinses, and the secondary antibody, a biotinylated goat anti-mouse antibody (from the Vectastain kit), was applied for 60 minutes at room temperature. After rinsing this with PBS-Tween, the avidin-biotin complex (ABC), also from the kit, was applied for 30 minutes at room temperature. After once again rinsing with PBS-Tween, a 3,3'-Diaminobenzidine (DAB) peroxidase substrate with metal enhancer (Sigma) was applied for eight minutes in the dark. The DAB tablet was vortexed in deionized water with the urea tablet supplied, just prior to application. After the DAB was removed, the cells are thoroughly rinsed with PBS to stop the reaction. Waste DAB and all pipettes and glassware were rinsed in bleach to neutralize them. The complex formed is highly stable and can be mounted for future observation.

Image Analysis Methods

Image Acquisition

Digital micrographs were obtained utilizing a Nikon Coolpix 995 digital camera to photograph stained cells at 400x magnification (40x objective) on a Nikon Eclipse TS100-F microscope. One important factor that was closely monitored was the saturation of the image. If the image should saturate in pixel intensity for red, green, or blue pixels, the data would be severely skewed. Images were stored in the camera’s memory card until downloaded. The image compression used was 1/4 Jpeg compression (“Fine” setting) with the image size set to 2048 x 1536.

Measurement Setup. Visualization of fluorescence associated with F-actin was accomplished by viewing through a fluorescence microscope with a filter cube selected
for FITC, and the neutral density filter in the open position to allow as much light as possible to reach the sample. Although it is generally advisable to leave the shutter only 50% open to reduce photobleaching, and compensate by increasing exposure times, Alexa Fluor 488 is extremely photostable as mentioned above. Since it was necessary to switch the trinocular head of the microscope so that the image was sent to the camera, it was easier to focus images using the camera’s LCD screen, which required the increased excitation to become visible, rather than repeatedly switching between the camera and the eyepiece. High power is critical to achieve resolution to clearly distinguish individual groupings of actin filaments. To acquire 400x images, it was necessary to flip the substrates over so that the side of the substrate containing the cells was on the bottom, closest to the objective. Twelve pictures, more or less, were taken for each structure on each substrate to provide sufficient data for analysis.

The Hoechst 33342 and ethidium homodimer-1 staining require images to be taken in a similar manner as for actin, with filter sets for DAPI and TRITC used for the Hoechst and ethidium, respectively. Images were taken in pairs, one through each filter, to provide a measure for cell viability. Pictures were taken at both 100x and 400x magnifications, for use by manual and automated measurements as described later. Twelve pictures were taken of each structure for each substrate at 400x, and two pictures were taken for each structure per substrate with the lower magnification objective.

The measurements for myosin did not require fluorescence. All images were acquired through bright field microscopy, without phase contrast. The same lamp intensity was used for all measurements. Substrates were also flipped because keeping them from drying required them to be covered in PBS, which could potentially spill due
to the depth required if they were not placed in this manner. Images were acquired at 100x magnification so that a large field of view could be used for the myosin density calculations. Four images were taken from each structure on each substrate with four images taken of the flat area surrounding each structure.

**Measurement of Alignment**

Accurate determination of cellular orientation is essential for assessment of the optimal parameters of channel width for cellular alignment. The images obtained for cells stained with phalloidin were used for measuring orientation of actin filaments, a known indicator for cell orientation.\(^{55,56,58}\) Regardless of its utility as a measurement of cell orientation, it is the orientation of the actin filaments which determines the axis of contraction, for which cell orientation is an approximation. Image analysis methods for determining orientations in biological images have received a large amount of study.\(^ {53,54,56,57,58,76,101,103,114,115,116,117}\) Nuclear orientation is also a good indicator of cell orientation.\(^ {52}\) Since cells are highly elongated, nuclei are constrained by the cell membranes, causing nuclear elongation in the direction of the cell elongation. Thus, orientation analysis of cell nuclei is also useful for determining cellular orientation.\(^ {118}\) Various methods for quantifying these orientations are discussed below.

**Manual Method.** The simplest method involves measuring the orientation manually of a large number of randomly selected stress fibers or nuclei. This can be done using ImageJ 1.26t (National Institutes of Health) to measure the angles between the perceived axis of orientation and the orientation of the channel wall. The most notable advantage of this method is that it allows high resolution angular measurements to be made. Unlike other methods discussed below, highly precise sub-degree orientation
measurements are attainable. However, this method also has several disadvantages. First, it is extremely tedious and slow, preventing analysis of a large number of samples per experimental group. Furthermore, it is susceptible to bias from the person analyzing the data, as is to a degree any such method relying on subjective measures. Because of this, the measurements are not entirely reproducible compared with other methods which produce the same result from the same input data without fail. Clearly this method is not attractive for analyzing large amounts of data, but it is useful for assessing the accuracy of the automated methods discussed later, as well as determining the orientation of the microchannels.

The manual method was performed as follows for measuring microchannel orientation. Each image was then opened with ImageJ, and the angle between the long axis of the microchannel and the horizontal axis was measured. This method could also be used for measuring actin filament orientations. In this case, the Orientation Angle (OA) index, previously defined as the difference between the orientation of the cell and the channel wall, was measured as follows:

$$OA = |\text{cell angle} - \text{wall angle}|$$

For instances where the above calculation was equal to or greater than 90°, 180° was first subtracted from the cell angle to account for this, allowing the formula to provide the correct acute angle between the cell and the channel wall.

The same procedure could be used for measuring the tissue culture control data, with the exception that there was no channel wall to act as a reference. Determination of the orientation angle for these cells was determined by calculating the resultant vector of
all the angles. The $x$ and $y$ components of the mean resultant vector were calculated as follows:

$$
\bar{x} = \frac{1}{n} \sum_{i} \cos \phi_i \quad \quad \bar{y} = \frac{1}{n} \sum_{i} \sin \phi_i
$$

In the above equation, $n$ represents the sample size, and $\phi$ represents the angle of each sample. The angle formed between the resultant and the $x$-axis is defined by the expression:

$$
\phi = \begin{cases} 
\tan^{-1} \left( \frac{\bar{y}}{\bar{x}} \right) & \text{if } \bar{x} > 0 \\
180^\circ + \tan^{-1} \left( \frac{\bar{y}}{\bar{x}} \right) & \text{if } \bar{x} < 0 
\end{cases}
$$

This is the angle substituted for the microchannel axis to obtain alignment measurements. It should be noted that *orientation* refers to the angle formed between the object to be measured and the horizontal axis, or for that matter any arbitrary axis, while *alignment* refers to the angle formed between the object and the axis of alignment, either the mean or microchannel axis.

**Blob Analysis of Cell Nuclei.** Blob, or connectivity, analysis is a method of isolating groups of connected pixels called “blobs,” which can then be subjected to further analysis such as measurement of orientation, elongation, area, etc. Pixels can have either 4- or 8-connectivity as shown below in Figure 30a and b, respectively. Pixels are considered to be connected if they are adjacent in the manner shown and have the same intensity. The type of connectivity required is determined by the intricacy of the pattern. For oval blobs, such as cell nuclei, 4-connectivity is sufficient. To begin the
analysis, images were converted to grayscale in Adobe Photoshop, and then to binary images by means of a threshold function, with the objects (blobs), whose orientations are to be measured, being white against a black background. Images were then resized to 256 x 192 pixels to speed analysis if the blobs are large with respect to individual pixels.

 Blob Analysis Procedure. A script was written in MATLAB (Appendix) to automate the analysis as follows, which is similar to other typical algorithms for 4-connectivity blob analysis. Images were imported into MATLAB and analyzed by column to locate areas of vertically connected pixels, or “columnar blobs.” An increase in blob number was incurred whenever the algorithm encountered a bright pixel following a dark pixel, as illustrated in Figure 31a. The value of the blob was multiplied by the pixel value (either 0 or 1), thereby setting the dark background to blob 0. These columns were then analyzed by row to locate horizontally connected columnar blobs, as demonstrated by Figure 31b. Next, blobs that were below a minimum area and those on the edge of images were excluded from further processing. Figure 32 shows the entire procedure with images from each stage of preprocessing, a-c, and each section of the MATLAB script, d-f.
Figure 31. (a) Vertical blob search and (b) horizontal blob search

Figure 32. Blob analysis method: (a) original image is converted to (b) grayscale by Photoshop and then to (c) binary image by a threshold function. The binary image is used by MATLAB to find (d) columnar blobs, coded by color, which are (e) connected horizontally. (f) Outlines of the blobs are superimposed on the original image.

The script produces a spreadsheet of data that indicates the area, centroid, orientation, and roundedness of each blob in the image, where $\text{pixel}_{x,i}$ and $\text{pixel}_{y,i}$ are the $x$
and \( y \) locations, respectively, of the \( i \)th pixel in the blob, and \( \text{pixel}_i \) is the value of the \( i \)th pixel in the blob. The centroid components and area, \( A \), are given as follows:

\[
\bar{x} = \frac{\sum \text{pixel}_{xi}}{\sum \text{pixel}_i} \quad \bar{y} = \frac{\sum \text{pixel}_{yi}}{\sum \text{pixel}_i} \quad A = \sum \text{pixel}_i
\]

These values allow calculation of the relative pixel locations of each blob with respect to its centroid.

\[
x_i = \text{pixel}_{xi} - \bar{x} \quad y_i = \text{pixel}_{yi} - \bar{y}
\]

Next, the product of inertia, \( P_{xy} \), and the moments of inertia with respect to the \( x \)- and \( y \)-axes, \( I_x \) and \( I_y \), are calculated, which are respectively equal to \( \sum y_i^2 \) and \( \sum x_i^2 \), used in the MATLAB script. These are used to calculate the angles, \( \phi_1 \) and \( \phi_2 \), the principal axes form with the \( x \)-axis.

\[
P_{xy} = \sum x_i y_i \quad I_x = \sum y_i^2 \quad I_y = \sum x_i^2
\]

\[
\phi = \frac{1}{2} \arctan \left( \frac{-2 P_{xy}}{I_x - I_y} \right)
\]

Because the downward directed \( y \)-axis in MATLAB is positive, it is necessary to correct for this by taking the negative of the angles; thus, \( \phi_1 \) and its perpendicular, \( \phi_2 \), are found as follows and reduced modulo \( 180^\circ \) as needed:

\[
\phi_1 = -\phi \pmod{180^\circ} \quad \phi_2 = \phi_1 - 90^\circ \pmod{180^\circ}
\]

Given these two angles, there are two values for the principal axis, \( I_u(\phi) \) (i.e., \( I_u(\phi_1) \) and \( I_u(\phi_2) \)), with the angle that minimizes the value being the major axis of orientation.

\[
I_u(\phi) = \frac{1}{2} (I_x + I_y) + \frac{1}{2} (I_x - I_y) \cos(2\phi) + P_{xy} \sin(2\phi)
\]
In the above equation, it was necessary to add the final term rather than subtract, because of the inverted axis in MATLAB. The product of inertia is sign dependent, whereas the moments of inertia, being squared, are not. The two values calculated are used to determine which angle is the angle formed by the major axis with the horizontal axis as follows:

\[
\begin{align*}
&< I_u(\phi_1) & \phi_1 \text{ is the major axis} \\
&I_u(\phi) > I_u(\phi_2) & \phi_2 \text{ is the major axis} \\
=& I_u(\phi_2) & \text{there is no major axis (blob is circular)}
\end{align*}
\]

Next, the roundedness is calculated from the major and minor axis lengths, which are defined as follows:

\[
\begin{align*}
\text{Major} &= \frac{2\sqrt{2}}{A} \sqrt{I_y + I_x + \sqrt{(I_y - I_x)^2 + P_{xy}^2}} \\
\text{Minor} &= \frac{2\sqrt{2}}{A} \sqrt{I_y + I_x - \sqrt{(I_y - I_x)^2 + P_{xy}^2}} \\
\text{Roundedness} &= \frac{\text{Minor}}{\text{Major}}
\end{align*}
\]

The quantity above\(^{116}\) is referred to as the roundedness (reciprocal of elongation) rather than as the shape index, as has been done previously, since the term shape index can also be used to refer to the alternate quantity below:\(^{120}\)

\[
\text{Shape Index} = \frac{4\pi \cdot \text{Area}}{\text{Perimeter}^2}
\]

Blob analysis provides a reproducible measurement of orientations that is relatively fast compared to manual analysis. Alignment can be determined by using the data obtained by the manual method for orientation of microchannels. There are several disadvantages, however. Overlapping blobs cannot easily be analyzed without providing
false orientation measurements. This is a problem for measuring the orientation of stress fibers which overlap many times with other fibers. Also, because binary images are required, it is highly susceptible to nonuniform fluorescence excitation, even with background subtraction. For this reason, blobs in darker areas will be smaller, resulting in a loss of filaments towards the image edges. Since the filaments are only a few pixels wide, whereas even minimal background will merge all filaments in the center of the image. For these reasons, this method is not acceptable for analysis of actin fiber orientation.

Blob analysis is highly useful for measuring nuclear orientation, however. Because care was taken to avoid growing cells past confluency, overlapping nuclei represent a fairly small percent of the total blobs to be analyzed. However, methods do exist for determining the number of objects to be counted even when they overlap, though they can increase computation time. The fluorescence images from which the nuclear orientations are determined show very low background staining, and even though there is some distortion in the binary images by the nonuniform fluorescence excitation, it does not affect the orientation calculations. Area calculations, though affected, are not of significant value outside of their use in calculating the orientation from the centroids, which is not influenced by this error at high resolutions. For these reasons, blob analysis was employed for determining the alignment of cell nuclei with the channel walls.

Two-Dimensional Fourier Transform. While blob analysis looks at the orientation of the connected pixels in an image, the two-dimensional fast Fourier transform (2D FFT) looks at the intensity gradients of an image. This method has been used often in digital image analysis to determine orientation distributions of various
types of fibers. Essentially, the image is a two-dimensional waveform. This can easily be visualized by examining an imaginary line drawn perpendicular to a series of parallel fibers as shown in Figure 33a. The intensity of the wave increases as it crosses the bright pixels of the fiber and decreases as it passes over the dark areas separating the fibers, as illustrated in Figure 33b. Thus, the frequency of this wave corresponds with the frequency at which the fibers repeat.

![Figure 33. (a) Profile across image of F-actin filaments stained with Alexa Fluor 488-phalloidin, and (b) intensity across profile](image)

The 2D FFT is calculated by taking the FFT in one direction (columns) and then the other (rows). The power spectrum of the 2D FFT contains information about the amplitude of waves at various frequencies and orientations, as shown in Figure 34. The center of the image contains the DC component (0 Hz) with the frequency increasing towards the edges of the images ($f_s/2$ Hz). The sampling frequency ($f_s$) is always 1 sample/pixel, with the number of samples corresponding to the image width.
One advantage to this method is its ability to provide orientation distributions for images of overlapping filaments. Furthermore, because grayscale images are used, it is not as susceptible to nonuniform fluorescence excitation or background staining as blob analysis is. One disadvantage is the unreliability of standard deviation calculations for images with randomly oriented fibers\textsuperscript{131}, limiting its usefulness in these situations. Also, the measurements of objects that are not extremely thin are affected because each object has orientation components in all directions in proportion to its width in that direction\textsuperscript{124}. However, its relative speed and robust nature make this the preferred method for measuring stress fiber orientations in situations with alignment distributions such as those predicted for muscle cells cultured on microchanneled substrates.

**2D FFT Procedure.** Images were first prepared for analysis by converting them to grayscale using Adobe Photoshop. Next, the images were cropped to produce square images with widths equal to $N$, where $N = 2^i$, and $i$ equals some integer, usually 10.
Images were then analyzed by a script in MATLAB by first windowing the image with a Gaussian window, modified from Palmer and Bizios,\textsuperscript{131} to prevent edge effects:

\[
g(y,x) = e^{\left(-\frac{100}{N^2} \left[\left(x-N/2\right)^2 + \left(y-N/2\right)^2\right]\right)}
\]

In the above equation, \(g(y,x)\) is the Gaussian window function, \(N\) is the picture width in pixels, and \(x\) and \(y\) are positional variables that refer to individual pixel locations. This equation is multiplied by the input image, Figure 35a, resulting in the modified image shown in Figure 35b. This is necessary because the 2D FFT treats the image as a repeating 2D waveform that repeats to infinity in all directions, otherwise causing discontinuities at the image edges. Since the edges are either at 0° or 90°, these orientations would contain large spikes, obscuring the filament orientations at these angles. After the image was windowed, it was possible to pad the image with zeros to produce a larger image as shown in Figure 35c, providing greater resolution. This was not done in practice, as images were cropped to 1024 x 1024 pixels. The DC component of the image, the mean, is then subtracted from all pixels.

![Figure 35](image-url)
Next, the 2D FFT is performed, which is a more efficient calculation of the 2D DFT for instances where \( N = 2^i \) as described above. The equation for the 2D DFT is given below:

\[
\mathcal{F}\{f(x, y)\} = \frac{1}{N} \sum_{x=0}^{N-1} \sum_{y=0}^{N-1} f(x, y) e^{-j2\pi(xu+vy)/N}
\]

The 2D FFT was then performed and the power spectrum obtained, as shown in Figure 36. Next, the 2D FFT data was converted to cylindrical coordinates to obtain values of intensities \( z \), at frequencies \( \rho \), and orientations \( \phi \).

\[
z(u, v) = |F(u, v)|^2
\]

\[
\rho(u, v) = \sqrt{(u - \frac{N}{2} - 1)^2 + (v - \frac{N}{2} - 1)^2}
\]

\[
\phi(u, v) = \tan^{-1}\left(\frac{v - \frac{N}{2} - 1}{u - \frac{N}{2} - 1}\right)
\]

One problem with this method becomes immediately evident. The coordinate conversion has limitations because pixels located near the center of the image can exist at fewer discrete angles than those further from the origin. As a result, low frequency components are not evenly distributed, producing very poor resolution. This can be remedied by two methods.
Convolution Mask. One method employs convolution masks, which are images of rectangles of unity intensity at various angles that are convolved with the power spectrum to obtain all the amplitudes at various orientations. To obtain 1\degree resolution, 180 convolution masks are required, which means 180 matrix multiplications per image. This is too slow for practical use with MATLAB, so resolution must be sacrificed for speed. Since orientation distributions might not differ by more than a few degrees between channel widths, resolution must be preserved, and therefore, this method is not acceptable for this application.

Bandpass Filter. Another method uses a bandpass filter to remove the low frequency components, where the resolution is poor, and the high frequency components, where image noise may be more of a problem. The low cutoff frequency is set at pixel 57 since this radius produces a circumference of approximately 360 pixels, which includes angles of all orientations when the coordinate conversion is applied. It is
necessary, then, to pad the image sufficiently so that the frequency corresponding to a radius of 57 pixels is lower than the lowest frequency associated with the filaments. Arbitrarily, the high cutoff frequency has been set at 1.25 times the low cutoff frequency, as that appeared to offer the best signal-to-noise ratio in tests. Once the 2D FFT power spectrum has been converted to cylindrical coordinates, the angles were rounded to the nearest degree to produce a histogram of amplitudes at various orientations, which corresponds to the orientation distribution of the actin filaments in that image as shown in Figure 37. This value for intensity is calculated as follows, where \( l \) and \( h \) are the lowpass and highpass cutoff frequencies, and \( M(u,v) \) is the bandpass filter convolution mask.

\[
I(\phi(u,v)) = z(u,v) * M(u,v)
\]

where \( M(u,v) = 1 \) if \( l < \rho(u,v) < h \),

\( else \quad M(u,v) = 0 \)

The data is then exported into a spreadsheet that can be imported into Excel for further processing. This allows calculation of the “orientation index” defined as follows:\(^{132}\)

\[
OI = 2 \left( \int_{-\frac{\pi}{2}}^{\frac{\pi}{2}} I(\phi) \cos(\phi)^2 d\phi \right) - 1
\]
Comparison of Two Orientation Analysis Methods. To demonstrate the differences and the similarities between the orientation distributions of nuclei and F-actin filaments, the component images (Figure 38 and Figure 39) of Figure 10 were analyzed by 2D FFT and blob analysis to produce the data below in Figure 40. The highest magnitude of the 2D FFT is found at approximately 150°, and corresponds well with the nuclear data. However, because the cell culture area to which the cells conform is a square, there is a significant percent of the actin filaments that parallel the edges, represented by the relative maxima at 88° and 179°. While there is some agreement between the data, it is clear that the actin filaments are fairly dispersed angularly, and they do not always align parallel with the nuclei. Furthermore, while the actin filaments
are affected by the edge of the pattern, the nuclei, located at some distance from the edge, are not so much affected, and thus apparently less likely to align with the pattern edge. Clearly, both measurements provide valuable information, and suggest that the underlying causes for nuclear and actin filament alignment may differ.

Figure 38. SMCs grown on adhesive square stained for DNA with Hoechst 33342

Figure 39. SMCs grown on adhesive square stained for F-actin with Alexa Fluor 488-phalloidin
Figure 40. Nuclear and F-actin orientation distributions as determined by blob analysis and 2D FFT analysis, respectively, of images in Figure 38 and Figure 39. Each bar represents the orientation of one nucleus.

Measurement of Cell Density and Viability

Three methods for obtaining cell counts from fluorescent micrographs of cell nuclei were considered for this research. The first method involves performing a manual count on a set of two-bit test images and calculating the number of fluorescent pixels in each image. Dividing the number of pixels by the number of cells counted yields a rough figure corresponding to the average number of pixels per cell nucleus. This method was highly susceptible to a number of inconsistencies in the data. The most problematic is that elongated cells have elongated nuclei with a potentially smaller projected area in the viewing plane, especially for cells growing on the sides of the channels. Thus, pictures with a large number of elongated cells may appear to have a
lower cell count than images with a similar number of highly disordered cells. Another
problem encountered was overlapping nuclei that resulted in an undercount in pixels,
which caused an underestimate in the cell count. Not allowing the cultures to become
confluent improves the situation somewhat, but this method cannot be relied on for
providing accurate data. Another problem was in developing a reproducible protocol for
enhancing the image that would not cause less focused cells to appear to contain fewer
pixels.

A second method for counting cell nuclei was a simple manual count. Images
were opened in Adobe Photoshop and subdivided into squares, facilitating cell
enumeration. Squares were chosen small enough to prevent overcounting or
undercounting. This method has some inherent advantages in that all cell nuclei would
be counted, including overlapping cells, whereas background noise would be less likely
to be counted than if an automatic cell counting system had been used. The obvious
drawbacks are that this type of count can be highly subjective, dependent on the
consistency of the analyst, and time-consuming. Regardless, this unbiased method is
probably the most versatile and necessary to demonstrate the accuracy of the automated
procedures.

The third method of nuclei counting was blob analysis. This proved to be the
most efficient method of nuclei counting. Although this eliminated cells due to overlap,
it was overall much faster and reproducible. Once an accurate cell count had been
accomplished for each picture, it was necessary to obtain a cell density for each of the
microscaffolds. Several acceptable definitions of what qualifies as cell density could be
argued for in this case. One would be the number of cells per image area, with the image
area calculated in square millimeters. Cell density can also be counted as the number of cells in a certain volume of the structure. This is simply a different way of stating the previous, and the two are proportional. Functionally, this may be the best definition, since for a tissue implant, the number of cells per total volume is the most important factor concerning cell density.

**Phenotype Analysis**

Phenotype analysis is an important measurement of cell function with both the quantities of actin and myosin being related to the cell’s contractility. Since it is hypothesized that cells cultured in microchannels will become more contractile, it is necessary to perform tests to measure the ratio of recognized markers of the contractile phenotype between the various structure types. Actin, which appears earlier in smooth muscle differentiation, is considered to be the less reliable of the two markers, as discussed earlier. However, actin associated fluorescence intensity is very simple to measure, and the images obtained for actin alignment needed no further processing. Cells stained with Alexa Fluor 488 conjugated phalloidin were viewed using a fluorescence micrograph and photographed as described above. The intensity of fluorescence per cell was calculated by dividing the average pixel intensity of the images by the number of cells per image as obtained by the cell density analysis. Because this method is sensitive to photobleaching, care was taken to protect the samples from light. As elaborated in the results concerning the PLL coverage, Alexa Fluor 488 is extremely photostable when compared to FITC, and was therefore selected as the fluorophore preconjugated with phalloidin. This is extremely important because variations in light
emission resulting from photobleaching will severely skew the pixel values measured. The measurements for myosin density were not affected by this parameter.

Images were acquired as described above and used as input for a MATLAB script designed to calculate red, green, and blue intensities for each image. All that was required by this script, listed in Appendix A, is to average the red, blue, and green matrices, and save them to a user specified file. These files were then opened in Excel for further processing. It is important to note that the red, green, and blue components do not correspond to any set combination of frequencies. For any given RGB combination, any number of spectra could be produced that would provide that result. Furthermore, because the cones in the human eye respond to all visible frequencies to varying degrees, the brightness is not simply the average of the three components. Because the values recorded are the intensities of the RGB components required to reproduce the original image as closely as is possible, the blue component (which is dimmest to the human eye) will contribute less to brightness than the same value for the green component. Thus, the camera converts the received image to RGB components and the monitor back again, using the following relationship for brightness:

\[
\text{Brightness} = 0.30 \text{ Red} + 0.59 \text{ Green} + 0.11 \text{ Blue}
\]

For the actin density analysis, it is not necessary to calculate this since only green intensities are compared, and no further analysis is required. However, for the myosin quantification, the value of the brightness was calculated as above. This produced a value for \(I\), the measured light intensity. The notation used here is \(I_0\) for illumination intensity, \(I_C\) for control image intensity, and \(I_S\) for sample image intensity. These data
were then used to calculate the average optical densities of the five structures. First, the optical density of a sample is known to be equal to the logarithm of its opacity, the inverse of transmittance:

\[ OD = \log \left( \frac{I_0}{I} \right) \]

The total optical density is the sum of the optical densities of all absorbing species which are each related by some constant to its concentration. There are essentially only two absorbing species to be considered. One is the “analyte,” the absorbance resulting from reaction of the DAB that is a consequence of the presence of the primary antibody directed against myosin. The “background” is taken to represent every other absorbing species, including DAB resulting from non-specific staining:

\[ OD = \log \left( \frac{I_0}{I} \right) = k_1|analyte| + k_2|background| \]

This is possible because optical densities are additive, and the background optical density can be assumed to be comprised of any number of components. Substituting the values \( I_s \) and \( I_c \) for \( I \) in the above equation, and realizing that since the control has no primary antibody and thus no specific DAB staining can occur, the \( k_1|analyte| \) term must be zero as follows:

\[ \log \left( \frac{I_0}{I_s} \right) = k_1|analyte| + k_2|background| \]

\[ \log \left( \frac{I_0}{I_c} \right) = k_2|background| \]
Combining equations and simplifying results in an expression for analyte concentration in terms of the intensities measured for the control and samples.

\[
|\text{analyte}| = \frac{\log \left( \frac{I_c}{I_s} \right)}{k_1}
\]

Although the constant \(k_1\) is not known, the data can be analyzed qualitatively, i.e. by normalizing the values obtained to the maximum mean. The values obtained were averaged over each substrate and then divided by the average cell density to describe the myosin density. To account for substrate-to-substrate variations, the values were divided by their flat surface counterparts. Once accomplished, these values could be compared.

**Statistical Analysis**

**Data Distributions**

There are two basic types of distributions of data present from this research: linear distributions, which follow a normal distribution, and circular distributions. Distributions around a circle, such as the direction of actin filaments, follow a bimodal distribution, since the axes are not directed, or vectorial. Differences in angles, such as between one sample and the mean, follow a linear distribution, as they are not continuous around a circle. Following visual inspection, all distributions which are not circular are assumed to be normal. Five circular data distributions, the von Mises, wrapped normal, wrapped Cauchy, wrapped exponential, and uniform, were investigated following data acquisition to determine the distribution that best fit the data. Both the von Mises and wrapped normal distributions have properties similar to the normal distribution for linear data, and
they are similar in form, whereas the wrapped Cauchy distribution is narrower in its dispersion as shown in Figure 41.

Figure 41. Probability densities for unimodal von Mises, wrapped Cauchy, and wrapped normal distributions given a mean orientation of 0° and mean resultant vector length of 0.70

The von Mises Distribution. All three probability density functions can be defined for either bimodal or unimodal data. The probability density function (pdf) for a bimodal von Mises distribution\textsuperscript{134} is given as follows, with \( I_0(\kappa) \) being equal to the modified Bessel function of the first kind of order zero, and \( \mu_0 \) is the mean direction, \( \kappa \) is the concentration parameter, and \( \rho \) is the length of the mean resultant vector of the population:

\[
M_2(\theta; \mu_0, \kappa) = \frac{1}{2\pi I_0(\kappa)} e^{\kappa \cos 2(\theta - \mu_0)}
\]

where

\[
I_0(\kappa) = \frac{1}{2\pi} \int_{0}^{2\pi} e^{\kappa \cos \theta} d\theta
\]

\[
\kappa = A^{-1}(\rho)
\]
The concentration parameter is determined by finding the value from a chart corresponding to the mean resultant length of the population.

The Wrapped Normal Distribution. Another circular distribution, the wrapped normal,\textsuperscript{135} has a pdf given by the equation for a bimodal distribution:

\[
W_z(\theta; \mu_0, \sigma) = \frac{1}{\sqrt{2\pi} \sigma} \sum_{k=-\infty}^{\infty} e^{ \frac{1}{2} \left( \frac{1}{\sigma^2} - \frac{1}{\rho^2} \right) (2\theta - \mu_0 + 2k\pi)^2} 
\]

where \[\sigma = \sqrt{-2 \ln \rho}\]

Both the wrapped normal and von Mises distributions are similar, although the von Mises may be preferable due to the types of statistical tests that may be performed on such distributed data.

The Wrapped Cauchy Distribution. The wrapped Cauchy distribution\textsuperscript{136} differs significantly from the wrapped normal and von Mises distributions because it has a much sharper peak for a given mean resultant length. The pdf for a bimodal wrapped Cauchy distribution is given below:

\[
C_z(\theta; \mu_0, \rho) = \frac{1}{2\pi} \frac{1 - \rho^2}{1 + \rho^2 - 2\rho \cos(2(\theta - \mu_0))}
\]

The Wrapped Exponential Distribution. The wrapped exponential distribution\textsuperscript{137} is useful because it provides for a sharp peak, unlike the other distributions studied as potential alternatives. The pdf for a bimodal wrapped exponential distribution is given below:

\[
E_z(\theta; \mu_0, \lambda) = \frac{\lambda e^{-2\lambda |\theta - \mu_0|}}{1 - e^{-2\pi \lambda}} \quad \text{where} \quad \lambda = \sqrt{\frac{\rho}{1 - \rho}}
\]
This function is valid over the range $\theta \in \left(-\frac{\pi}{2}, \frac{\pi}{2}\right)$.

**The Uniform Distribution.** The uniform distribution is constant for all values of $\theta$. The uniform distribution should be the expected distribution for random orientation distributions. The pdf for the uniform distribution is given by:

$$U(\theta) = 1$$

**Linear and Circular Angular Distributions**

To avoid confusion, $\phi$ refers to angular samples, and $\theta$ refers to the population. Both are distributed around an arc (i.e. circular data). On the other hand, $\Delta \phi$ and $\Delta \theta$ refer to differences between two angles (i.e. linear data). To remain consistent with conventional practice, theoretical mean orientations for circular populations are denoted by $\mu_0$, while sample means are denoted by $\bar{\phi}$. While calculations such as means are straightforward with linear data, the same methods cannot be applied to circular data. As was mentioned before, the mean angle from a set of data is not simply the arithmetic mean, but rather the angle formed with the $x$-axis by the resultant of all the angles, treated as vectors and placed end to end, the resultant vector. Other problems arise when circular distributions are bivariate. Rather than a set of data that is a distribution of angles, the data from the 2D FFT analysis is a distribution of angles with varying magnitudes. Thus, either the data must be treated as a bivariate, bimodal circular distribution, or it must be altered to fit a unimodal univariate circular distribution.
Data Transformation

There are two basic issues that must be resolved when converting the data into the form required by the statistical tests used. The first, most basic transformation, is used to transform the bimodal distribution of the axial data into a unimodal distribution. This is done by a process of angle doubling, where the angles are doubled so that they cover a 360° arc and axes measured as 180° apart are thus equated. When angles are doubled, the angles must be reduced modulo 360° so that they are on a range of 0°-360°. All angles must be doubled by this method, and the resulting data, either mean angles or standard deviations, must be halved to transform the angle back to the 180° range of the original data.

A more complex transformation is required to adjust data from a bivariate to a univariate distribution. A potential method treats a sample at angle $\phi_i$ and magnitude $M_i$, as being equivalent to $M_i$ samples at angle $\phi_i$ with unity magnitude. A further adjustment is required, however, so that the number of samples is not also increased, as this would incorrectly affect measures such as the standard deviation. For this reason, the method used here is the same as the above except that the number of resulting samples is not $M_i$, but $M_i$ divided by the sum of magnitudes and multiplied by the total sample size. This is better illustrated below, where the calculations for mean and standard deviations of linearly distributed angles are given for both the univariate (left), and transformed bivariate (right) cases.
\[
\Delta \phi = \frac{\sum_i \Delta \phi_i}{n}
\]

\[
\Delta \bar{\phi} = \frac{\sum_i \left( \frac{M_i}{\sum_i M_i} \right) \Delta \phi_i}{\sum_i \left( \frac{n}{\sum_i M_i} \right)}
\]

univariate  
bivariate

In the case on the left, \(i\) ranges from 1 to \(n\), the number of samples, whereas on the right, in the bivariate case, \(i\) ranges from 1 to \(q\), the number of histogram bins, and \(n\) is the number of samples. The transformation of the bivariate case takes into account that a sample at angle \(\phi_i\) with magnitude \(M_i\), is equivalent to \(M_i\) samples at angle \(\phi_i\) with unity magnitude. However, this alters the sample size. Even taking the range of \(i\) to be equal to \(q\), and thus 180 is not correct, as the data obtained from the MATLAB script actually sums magnitudes when producing the angular distribution. The true sample size must be recovered by multiplying by \(n\), the true sample size, and dividing by the sum of magnitudes. Thus, the term \(c\)

\[
c = M_i \sum_i \frac{n}{M_i}
\]

is equivalent to the number of samples at angle \(\Delta \phi_i\). The equation for mean angle can actually be simplified to the equation below by removing the constant portion of \(c\) which divides out leaving:

\[
\Delta \bar{\phi} = \frac{\sum_i (M_i \Delta \phi_i)}{\sum_i M_i}
\]
The standard deviations also must be adjusted by the same method, and result in the following equation, with the standard univariate case shown at left for comparison with the transformed, bivariate case on the right:

\[
\begin{align*}
\text{univariate} & \quad s = \sqrt{\frac{\sum (\Delta \phi_i - \bar{\phi})^2}{n-1}} \\
\text{bivariate} & \quad s = \sqrt{\frac{\sum M_i \sum \frac{n}{M_i} (\Delta \phi_i - \bar{\phi})^2}{n-1}}
\end{align*}
\]

This is similar to the case for the mean, for here the number of differences between a sample angle, and mean angle must be transformed. Therefore, for each angle, \(\Delta \phi_i\), there are \(c\) squared differences. The number of samples can thus rightly be denoted as \(n - 1\).

A similar situation follows for circularly distributed angles where the mean angle for the univariate and bivariate cases can be determined by the equations below for the mean resultant components of the doubled angles. The subscripts, 2, on the resultant components denotes they refer to the resultant of doubled angles, \(2\phi\). The equations could be used for unimodal data by replacing the \(2\phi\) term with \(\phi\):

\[
\begin{align*}
\bar{x}_2 &= \frac{\sum \cos 2\phi_i}{n} \\
\bar{y}_2 &= \frac{\sum \sin 2\phi_i}{n}
\end{align*}
\]

\[
\begin{align*}
\bar{x}_2 &= \frac{\sum M_i \sum \frac{n}{M_i} \cos 2\phi_i}{n} \\
\bar{y}_2 &= \frac{\sum M_i \sum \frac{n}{M_i} \sin 2\phi_i}{n}
\end{align*}
\]
or\[\bar{x}_2 = \frac{\sum (M_i \cos 2\phi_i)}{\sum M_i} \quad \bar{y}_2 = \frac{\sum (M_i \sin 2\phi_i)}{\sum M_i}\]

bivariate

For circularly distributed data, the circular variance, $s^2$, and circular standard deviation, $s$, in radians are calculated as follows, where $l$ is the number of modes, being two for the bimodal case:

\[
s^2 = \frac{2(1-r)}{l^2}
\]

\[
s = \frac{\sqrt{-2 \ln r}}{l}
\]

Statistical Tests

**Goodness of Fit Testing.** Testing for goodness of fit was only performed on the axial data, to test if the probability density functions were good predictors of the sample data. Testing was performed using a $\chi^2$ test defined as follows where $o$ and $e$ are the observed and expected values, respectively:

\[
\chi^2 = \sum \frac{(o_n - e_n)^2}{e_n}
\]

The data points were binned so that there was a minimum of five points per bin. $P$ values were generated from a chart based on the $\chi^2$ values obtained. Testing was essential because the types of statistical tests available were dependent on the distribution of the data.

**Student’s $t$ Test.** The most common statistical test performed for data obtained from the experiments in this research is the Student’s $t$ test. Tests were performed in
Excel when possible for two samples with an unknown and unequal variance. *P* values were obtained for two-tailed tests, enabling determination of the level of significance of the data. The test was repeated for every combination of treatments (e.g. myosin density in 20 µm vs. 40 µm wide microchannels) and a table formed of the results. This test was performed for a wide range of data from cell density measurements to metrology data. For instances such as the actin data where the data needed to be manipulated to calculate the correct values for standard deviation and mean, the Student’s *t* test was calculated manually using the statistic for large sample sizes:

\[
t = \frac{(\Delta \bar{\phi}_1 - \Delta \bar{\phi}_2) - (\Delta \bar{\theta}_1 - \Delta \bar{\theta}_2)}{\sqrt{s_1^2 + s_2^2}}
\]

*V* Test. The *V* test,\(^{139}\) or modified Rayleigh test, was applied to axial data from nuclear and actin orientation measurements. In order to account for the bimodal distribution of the data, the data were taken only over the range of \(0^\circ \leq \phi < 180^\circ\), and doubled, so that a continuous unimodal \(360^\circ\) distribution would be present. The test statistic, for the modified Rayleigh test is:

\[
V = \sqrt{2n\theta}
\]

In the above test statistic, *n* is the number of angular values, and \(\theta\) is defined by the equations that follow, where *r* is the length of the sample mean resultant vector. The theoretical angle about which the data cluster, \(\mu_0\), is the angle of the microchannels with the horizontal image axis. Because the angles, \(\phi\), were doubled for obtaining the components of the mean resultant, the value of the mean angle must also be doubled.
\[ \theta = r \cos(2\phi - 2\mu_0) \]
\[ r = \sqrt{x_2^2 + y_2^2} \]
\[ \tan^{-1}\left(\frac{y_2}{x_2}\right) \quad \text{if } x_2 > 0 \]
\[ 2\phi = 180^\circ + \tan^{-1}\left(\frac{y_2}{x_2}\right) \quad \text{if } x_2 < 0 \]

Using the value obtained for \( V \), and comparing it to values obtained from the chart for various levels of significance, \( \alpha \), the null hypothesis, that the angular distribution is random, can be tested. This simple test essentially investigates whether the clustering of data is due to chance or is a result of other causes, such as microchannel width.

**Rayleigh Test.** For instances where no theoretical mean angle could be deduced (i.e. for cells cultured on either flat surfaces or from in vivo tissue samples), it was necessary to perform a less powerful statistical test. In these instances, the Rayleigh test\(^{138} \) was used, for which the test statistic was:

\[ z = nr^2 \]

In the above statistic, \( n \) is the number of samples, and \( r \) is the mean resultant length, calculated by doubling the angles, as before. This is a modification used for large sample sizes. \( P \) values were calculated using the following formula:\(^{136} \)

\[
P = e^{-z} \left[ 1 + \frac{2z - z^2}{4n} - \frac{24z - 132z^2 + 76z^3 - 9z^4}{288n^2} \right]
\]

This formula can be simplified to \( P = e^{-z} \) for sample sizes of 50 or greater.
Analysis of Variance of Circular Data. Analysis of variance was performed on circular data from both the nuclear and actin alignment experiments. First, the mean and variance of the data were calculated as described above, and these values were analyzed by the SAS statistical software package. The program code used to perform the statistical analysis is shown in Appendix B.

Bonferroni Correction

An adjustment is necessary when performing multiple comparisons. To compare every combination of $n$ channel widths means $n(n-1)/2$ comparisons. For five channel widths and one flat control surface, 15 comparisons are required. When more than one comparison is made, an adjustment is needed. For Student’s $t$ tests, the Bonferroni correction is applied by multiplying the calculated $P$ value by the number of tests run.
CHAPTER 3

RESULTS

Characterization of Scaffolds

Phase Contrast Microscopy

PDMS substrates were viewed following mold release to ensure that they were properly formed and did not tear or possess other structural defects that would be clearly visible through a microscope. Pictures taken of each of the five structures used for cell culture are presented below in Figure 42. The images clearly show that the substrates are relatively free from defects. It is problematic to accurately measure the dimensions of the channels accurately by phase microscopy due to difficulties in focusing on the structures to produce a clear image. As a result, further tests are necessary to verify if the dimensions of the channels of the PDMS structures are the same as those on the silicon wafer, the mask, and the mask pattern designed in AutoCAD.
Figure 42. Phase contrast images of PDMS microchannel scaffolds of (a) 20 µm, (b) 30 µm, (c) 40 µm, (d) 50 µm, and (e) 60 µm widths

Roughness Step Tester Metrology

The processed silicon wafer used to mold the PDMS substrates and one resulting PDMS mold were subjected to analysis by a WYKO RST (Veeco Instruments). The depth of one channel in each microscaffold was measured by determining the depth of the channel on the PDMS or, alternatively, the depth of the channel wall, since the Si wafer possesses the inverse structure of the PDMS scaffold. The data obtained show a wafer etch depth of 16.988 µm ($s = 0.745$) for five samples. This compares to a PDMS channel depth of 15.216 µm ($s = 2.63$) for five samples. The higher standard deviation for the PDMS is probably a result of the difficulty of the RST to accurately measure depths on the transparent scaffold. These are both within the desired range of 10 to 20 µm. In order to assure that etch depth was not significantly dependent on the location of the area
exposed to the etchant, since any variation in depth might influence final cell density, channel depths were plotted against channel widths as shown in Figure 43.

Figure 43. Channel depth as measured by RST

Surface Profiler Metrology

The data from the surface profiler (KLA-Tencor) analysis proved inadequate to determine the actual channel width, as documented below in Figure 44. Due to the size of the profiler stylus, it was not capable of determining the actual depth, since the stylus would apparently not fit in the channels. Also, due to the slope of the sides of the stylus, it was not possible to accurately measure the location of the sides of the channels; thus, the channel widths are shown erroneously to vary with depth, preventing any reliable width measurements.
Figure 44. Surface profiler data for 40 µm wide microchannel structure

Scanning Electron Microscope
Metrology

To get accurate data for channel depths and widths, one PDMS substrate was sliced perpendicularly to the channels to expose a cross-section of each structure area, and was sputter coated with palladium for viewing with an SEM (Amray), as shown in Figure 45 and Figure 46.

Figure 45. Sputtered PDMS scaffold cross section
Electron micrographs were taken and analyzed with ImageJ to determine the widths and depths of the channels, shown in Figure 47.
The data show much less variance than that obtained from RST measurements, resulting in the low standard deviation as shown. Significance testing was performed using a Student’s $t$ test for two samples with unknown and unequal variances. The $P$ values following application of the Bonferroni correction are shown in Table 1. Results show no significant distance between any of the channels using a significance level of $\alpha = 0.01$ prior to applying the Bonferroni correction. All combinations of channel widths were compared because of the possibility of an effect of location of the structure on the wafer might have on etch depth.

Table 1. $P$ values for $t$ test for means of channel depth following Bonferroni correction

<table>
<thead>
<tr>
<th></th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 µm</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 µm</td>
<td>1.000</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µm</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>60 µm</td>
<td>1.000</td>
<td>1.000</td>
<td>0.31852</td>
<td>1.000</td>
</tr>
</tbody>
</table>

The data reported above are important because they confirm the fidelity with which the microfabrication processes produced the Si wafer and the PDMS was cast against the wafer to produce the silicone substrates. Without a high degree of consistency within and between microscaffolds, the data could not be reliably compared, since cell density, attachment, and phenotype are, or may be, highly susceptible to differences in surface roughness, etch depth, and channel width. In short, without a precise, uniform cell culture substrate system, reliable data cannot be obtained, and accurate conclusions may not be drawn.
Quantitative Analysis of PLL Coverage

To demonstrate the uniformity of PLL adhesion, one PDMS substrate was coated with fluorescein isothiocyanate (FITC)-labeled PLL instead of the unlabeled PLL used for cell culture. The FITC-PLL fluoresces when irradiated by 480 nm light, and micrographs of the scaffolds provide an efficient means of comparing the thickness of coverage between culture microscaffolds (comparison of means of pixel intensities) as well as a comparison of the uniformity of coverage in the different microscaffolds (comparison of variance of pixel intensities).

Photobleaching was a major problem encountered during analysis, causing those structures which were photographed first to have the highest green intensities, as seen in Figure 48. Structures are denoted by Roman numerals as shown in Table 2. Pictures were taken of structure VI first, which explains why it is brightest. Pictures followed from I–V, and then VII–X. Due to the placement of the structures on the PDMS substrate (I–VI on row 1 and VII–X on row 2), some of the structures were exposed for much longer times than others, prohibiting any determination from being made from the average intensities. However, all the structures show higher green values than either the FITC-PLL layered PDMS, or the FITC-PLL layered glass slide. This demonstrates the effectiveness of the polyelectrolyte multilayers in promoting adhesion of PLL. Finally, both the unlayered glass and the PDMS layered with unconjugated PLL show average green intensities of zero, proving that the fluorescence observed is indeed most likely coming from the FITC conjugated with PLL.
Figure 48. Average green intensities of images

Table 2. Structure dimensions

<table>
<thead>
<tr>
<th>Structure</th>
<th>Channel Width (µm)</th>
<th>Channel Wall Thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>II</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>III</td>
<td>30</td>
<td>5</td>
</tr>
<tr>
<td>IV</td>
<td>30</td>
<td>10</td>
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<tr>
<td>V</td>
<td>40</td>
<td>5</td>
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<tr>
<td>VI</td>
<td>40</td>
<td>10</td>
</tr>
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<td>VII</td>
<td>50</td>
<td>5</td>
</tr>
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<td>VIII</td>
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<td>10</td>
</tr>
<tr>
<td>IX</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td>X</td>
<td>60</td>
<td>10</td>
</tr>
</tbody>
</table>

For accurate determination of whether the channels limit diffusion of the PLL, possibly by trapping air bubbles in the channels, or increase layer thickness by making rinsing more difficult, it is necessary to compare the green intensity values of the channel.
bottoms and the tops of the channel walls. Both these values are highly consistent as seen above in Figure 48, and more clearly below in Figure 49. For the most part, especially in the structures with 10 µm thick channel walls for which the wall top fluorescence was easier to measure (and the only structures ultimately used), there appears to be no trend, indicating that PLL adhesion is uniform throughout the substrate. This is important because thicker or more confluent coverage of PLL in wider microchannels may increase the cell density supported in those structures, mediated by a potentially more adhesive surface film.

![Graph showing ratio of green intensity in channel bottoms to channel wall tops for 5 µm and 10 µm channel walls across different channel widths.](image)

**Figure 49.** Relative green intensity of fluorescence from channel floors and channel wall tops

The null hypothesis that there was no difference in FITC-PLL thickness was tested by a Student’s $t$ test. A table of values shown in Table 3, gives the $P$ values indicating the significance levels, $\alpha$, at which the two samples can be presumed different.
It was earlier noted the difficulty of measuring the green intensity at the tops of the 5 µm thick channel walls, and since only the 10 µm thick channel wall structures were used, any difference in PLL coverage for various channel widths on the 5 µm thick channel wall structures is not important. The results show that it is unlikely that there is an inconsistency in the PLL coverage.

Table 3. *P* values for significance testing of PLL uniformity (*t* test with *n*=5) following Bonferroni correction

<table>
<thead>
<tr>
<th></th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 µm</td>
<td>1.0000</td>
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<td></td>
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<tr>
<td>40 µm</td>
<td>0.1946</td>
<td>1.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µm</td>
<td>1.0000</td>
<td>1.0000</td>
<td>0.0726</td>
<td></td>
</tr>
<tr>
<td>60 µm</td>
<td>1.0000</td>
<td>1.0000</td>
<td>0.7634</td>
<td>1.0000</td>
</tr>
</tbody>
</table>

**Cell Density**

The cell density measurements were designed to ensure that the structures were populated by a sufficient quantity of cells to produce a confluent culture. Pictures of cells taken under phase contrast microscopy show that nearly the entire channel surface was covered by cells, though this was not quantified. Images of cultures on each of the structures are shown below in Figure 50.
Figure 50. Phase contrast images of cells cultured in (a) 20 µm, (b) 30 µm, (c) 40 µm, (d) 50 µm, (e) 60 µm wide PDMS microchannels and on flat PDMS substrates

Cell density was measured using two different methods as described, depending on the magnification used. All cells were stained with both ethidium homodimer-1 and Hoechst 33342, with example fluorescence micrographs shown in Figure 51. Images taken using the 40x objective were enumerated by the MATLAB blob analysis script as they were analyzed for nuclear orientation. The 10x images, while encompassing a larger area for more cells and enabling a better measurement, were not of sufficient resolution to permit reliable automatic analysis. For this reason, the manual counting of cells as described previously was undertaken.
Figure 51. Fluorescence micrographs of cell nuclei stained with (a, d) Hoechst 33342 and (c, f) ethidium homodimer-1. Images were taken at both (a-c) 100x and (d-f) 400x magnifications, and used for determining cell density, viability, nuclear orientation, and nuclear roundedness. Images (b) and (e) are digitally combined images of (a, b) and (d, f) respectively, to highlight dead cells, violet. All images were enhanced to show contrast.

The data for the blob analysis measurement of cell density show a much higher cell total (combined live and dead) for the 30 µm wide channels than for the other substrates as shown in Figure 52 and Figure 53. The data obtained were converted to cell density by dividing by the area of each picture. Cells were seeded at an initial density of 100 cells/mm², resulting in an approximately three- to four-fold increase in total cell density during six days of culture.
Figure 52. Manual count of cells from 100x magnification images of cell nuclei stained with Hoechst 33342 and ethidium homodimer-1.

Figure 53. Blob analysis count of cells from 400x magnification images of cell nuclei stained with Hoechst 33342 and ethidium homodimer-1.
The two methods of counting cells differ widely in their results, with most of the difference being in the count of the cells stained by Hoechst 33342. The standard deviations for both cell counting methods are high, with that for the automated count being considerably higher, possibly due to clustering of cells in the smaller image field. The two methods are graphically compared in Figure 54. It should be noted that for both image sets, the images were taken in pairs, one image for ethidium and one for Hoechst; thus, there should not be any difference in the ratio of the two counts (Hoechst and ethidium) for each method. However, such a difference does exist, and it is reflected in the varying calculations for cell viability by the two methods, as depicted in Figure 55.

![Figure 54. Comparison of cell counts by different methods for 100x images (manual count) and 400x images (automated blob analysis count)](image-url)
Student’s $t$ tests were performed to determine the statistical significance of the differences in mean cell density and viability for the various structures. Tables of $P$ values are presented below in Table 4 and Table 5. With more samples in the blob analysis cells counts, the differences are more significant. However, with both methods, the statistics show that the 30 µm wide channels (the most highly populated) have significantly more cells than the 40 µm channels (which have the fewest cells). For the automated analysis, the 30 µm wide channels have more cells than all other channels.
The viability statistical tests are displayed below in Table 6 and Table 7. The results from the manual counting of cells from the 100x images do not indicate any variation in cell viability between structures with channels of different widths. The blob analysis results do indicate a significant difference in cell viability for structures of different channel widths. Namely, there appears to be an increase in viability for cells grown in the narrower channeled substrates. Although not significant for the 100x manually tabulated cell counts, there is a trend in both data sets towards increased viability for cells grown in channeled substrates. However, there is not much of a noticeable difference between the narrower channels.
Table 6. *P* values for Student's *t* test for comparison of mean viability of cells from channels of different widths for 100x images following Bonferroni correction

<table>
<thead>
<tr>
<th>Width (µm)</th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 µm</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 µm</td>
<td>1.0000</td>
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<td></td>
<td></td>
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<tr>
<td>50 µm</td>
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<td>1.0000</td>
<td>1.0000</td>
<td></td>
<td></td>
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<td>1.0000</td>
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<td></td>
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<tr>
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</tr>
</tbody>
</table>

Table 7. *P* values for Student's *t* test for comparison of mean viability of cells from channels of different widths for 400x images following Bonferroni correction

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<thead>
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<th>40 µm</th>
<th>50 µm</th>
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<tr>
<td>50 µm</td>
<td>1.0000</td>
<td>1.0000</td>
<td>1.0000</td>
<td></td>
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</tr>
<tr>
<td>60 µm</td>
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<td>0.024432</td>
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<tr>
<td>Flat</td>
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<td>1.0000</td>
<td>0.534984</td>
<td>0.7259265</td>
<td>1.0000</td>
</tr>
</tbody>
</table>

**Comparison of Counting Methods**

Table 8 shows *P* values for the comparison of mean total cell density and cell viability between the two different counting methods. The results show that the two methods differ widely. The possible reasons for the differences are discussed later; however, a small number of pictures at 400x magnification were enumerated both manually and automatically, displaying a mean error of 7.15% (*n*=8) and 48.29% (*n*=3) between the two methods for the total and dead cell counts. Although the former measurement is probably acceptable, the latter is not.
Table 8. *P* values for levels of significance of equality of mean total cell density and viability for the manual and blob analysis cell enumeration methods following Bonferroni correction

<table>
<thead>
<tr>
<th></th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
<th>Flat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Cell Density</td>
<td>0.0025855</td>
<td>0.027535</td>
<td>0.31400</td>
<td>0.0071825</td>
<td>1.293E-04</td>
<td>0.004769</td>
</tr>
<tr>
<td>Cell Viability</td>
<td>0.0023915</td>
<td>0.001279</td>
<td>0.0021435</td>
<td>0.00915</td>
<td>0.46765</td>
<td>0.39109</td>
</tr>
</tbody>
</table>

To illustrate, two sets of images shown below in Figure 56 were formed by combining the DAPI and TRITC fluorescence images with the corresponding phase contrast image. The automated count of cells for the two image sets are one for both the dead cell nuclei counts (red) and 26 and 70 for the total nuclei counts (blue) for the left and right pictures, respectively. A manual count gives a total of 26 and 69 total cells, and one and five dead cells for the two data sets. As can be seen below with the aid of the phase contrast image superimposed upon the fluorescence image, the ethidium homodimer-1 stains the RNA significantly causing adjacent cells to become joined during the image processing, resulting in a dramatic undercount.

Figure 56. Combination of phase contrast and fluorescence images of cells in (a) a sparse portion of a culture, and in (b) a dense portion of a culture
Cell Phenotype

Actin Density

Measurements for actin density were performed by dividing the mean green intensity of images of cells stained for F-actin (Figure 57) by the number of cells calculated per structure to give a ratio of actin densities between cells grown in various structures. Because the intensity of the Alexa Fluor 488 was to be measured quantitatively and used to make comparisons between different structures on the same substrate, it was necessary to prove that the Alexa Fluor 488 did not substantially photobleach during the data acquisition under the light intensities used.

Figure 57. Fluorescence micrographs of smooth muscle cells stained with Alexa Fluor-488 conjugated phalloidin cultured on (a) flat PDMS and (b-d) PDMS microchannels
Pictures were taken every 30 seconds for 10 minutes at maximum excitation and the results graphed in Figure 58. The final measurement at 10 minutes was actually at a higher intensity than the initial measurement, indicating that random fluctuations in bulb intensity had a greater effect than any potential photobleaching. However, the difference between the maximum and minimum was only 0.53% of the initial intensity, which should be less than any measurable difference in fluorescence intensity associated with a change in actin density.

![Figure 58. Measurement of change in fluorescence image intensity over 10 minutes constant excitation using fluorescence microscope with neutral density filter open](image)

The intensity of green fluorescence was measured from digital micrographs of F-actin filaments stained with Alexa Fluor 488 conjugated phalloidin. The mean intensities of the green component of the images, both per cell and per picture are graphed in Figure
The graph clearly illustrates that the intensity of the fluorescence does not appear to be proportional to cell density. Significance testing by Student’s $t$ tests (Table 9) shows that, with the exception of the cells cultured on tissue culture plastic, there is no consistent significant difference in the mean intensities per image. The fluorescence difference involving the tissue culture plastic may reflect a lower intrinsic fluorescence of the substrate with respect to the PDMS. Testing by $t$ test on the “actin density,” or green intensity per cell, shows that the means are not equal, as shown in Table 10.

Figure 59. Fluorescence from Alexa Fluor 488 conjugated to phalloidin bound to F-actin in cells cultured in various width microchannels
Table 9. *P* values obtained for significance testing for equality of means of green intensity for structures of various channel widths

<table>
<thead>
<tr>
<th></th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
<th>flat</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
</tr>
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</tr>
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<td></td>
</tr>
<tr>
<td>TC</td>
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<td>0.029662</td>
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</table>

Table 10. *P* values obtained for significance testing for equality of mean green intensity per cell for structures of various channel widths following Bonferroni correction

<table>
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**Myosin Density**

Measurement of myosin density per cell is perhaps the best method of quantifying cell phenotype, since myosin is essential for a cell’s ability to contract and begins to be expressed relatively late in fetal development\(^{10}\). The images shown in Figure 60a and b demonstrate the staining for myosin, from which it is clearly evident that the channels cause elongation and cellular alignment with the microchannels. These were taken at a higher camera zoom setting that the pictures for automated quantitative analysis in order to more clearly show the myosin staining pattern. Figure 60c and d are images used for image analysis, which are at a lower magnification to provide a larger field of view. The control on the left, Figure 60c, differs from the image on the right, Figure 60d, only in that no primary antibody directed against myosin was applied, so that the amount of non-
specific staining could be determined. There are several factors that contribute to the optical density of the sample. These include the PDMS substrates, especially the channel wall edges, the cells, and DAB (both specific and non-specific, i.e. background, staining).

Figure 60. Bright field image of RASMC on PLL-layered (a) flat PDMS, and (b) 20 µm wide channeled PDMS, stained for smooth muscle myosin (400x magnification). Also, (c) control, and (d) experimental group, for 40 µm wide PLL-layered PDMS microchannels stained for smooth muscle myosin (100x magnification)

The method employed for image analysis utilized a MATLAB script that analyzed each RGB image for average red, green, and blue intensities. These data were stored in spreadsheet form, and the appropriate calculations applied as discussed in the
section on phenotype analysis. The values collected for $I_S$ and $I_C$, the intensities of light for the sample and control collected, respectively, are shown in Table 11.

![Table 11. Raw light intensities as calculated by MATLAB](image)

<table>
<thead>
<tr>
<th></th>
<th>II 20 µm</th>
<th>IV 30 µm</th>
<th>VI 40 µm</th>
<th>VIII 50 µm</th>
<th>X 60 µm</th>
<th>Flat ∞</th>
</tr>
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<td>$I_S$</td>
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<td>157.737</td>
<td>160.331</td>
<td>164.657</td>
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<td>141.144</td>
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<td>165.865</td>
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<table>
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<th></th>
<th>II 20 µm</th>
<th>IV 30 µm</th>
<th>VI 40 µm</th>
<th>VIII 50 µm</th>
<th>X 60 µm</th>
<th>Flat ∞</th>
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</thead>
<tbody>
<tr>
<td>$I_C$</td>
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<td>169.527</td>
<td>168.939</td>
<td>173.168</td>
<td>175.886</td>
</tr>
</tbody>
</table>

Plotted (Figure 61), this clearly shows that the optical densities of the test samples are clearly higher in all cases than the control samples, indicating the detection of myosin.

![Figure 61. Average light intensities plotted against microchannel width](image)
These data were then used to calculate the relative optical densities for the specifically reacted DAB in the samples, normalized to the maximum average measurement. During staining, one of the PDMS wells designed to contain the liquid leaked, probably due to the PBS-Tween in which the cells were rinsed. These data were excluded from further analysis because the substrate did not conform in staining method to the other samples. These remaining data, with the associated standard deviations, are charted in Figure 62.

![Figure 62. Ratio of myosin densities in cells grown in structures to cells grown on flat surfaces](image)

The high variability is due primarily to the low number of samples \((n=2)\). Another potential factor is the method of obtaining optical densities from RGB images taken by a digital camera. Although there may appear to be a trend in the data, a decrease in myosin density with channel width increase, it was not found to be statistically significant enough to reject the null hypothesis that the samples had equal means following a Bonferroni correction at any significance level.
Cell Alignment

Actin Fiber Alignment

Actin fiber alignment was measured by two-dimensional FFT as described earlier. The data obtained from the MATLAB script consisted of a distribution of magnitudes from the power spectrum of the 2D FFT at angles from 1° to 180° as shown in Figure 63.

![Figure 63. Actin filament alignment distribution](image)

A value for the orientation index was calculated as a rough measure of whether the filaments were aligned or random. These data are shown in Figure 64 and indicate a trend towards alignment in narrow microchannels and towards random orientations in wider microchannels and on flat substrates. A Student’s $t$ test was performed to determine if the mean Orientation Indices were different for the various structures. The
results in Table 12 show that there is indeed a significant difference between the mean Orientation Index between channel widths. The greatest rate of change in the index occurs for the narrower channels, with the wider channels not experiencing as noticeable an effect.

Figure 64. Orientation Index for F-actin filaments in cells cultured on different structures

<table>
<thead>
<tr>
<th>Channel Width</th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
<th>Flat</th>
<th>TC</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 µm</td>
<td>1.0000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 µm</td>
<td>0.066221</td>
<td>0.40056</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µm</td>
<td>0.15647</td>
<td>1.0000</td>
<td>1.0000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 µm</td>
<td>0.22091</td>
<td>0.53174</td>
<td>1.0000</td>
<td>1.0000</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Flat</td>
<td>2.2365E-05</td>
<td>1.0303E-03</td>
<td>0.072080</td>
<td>0.080207</td>
<td>0.23935</td>
<td></td>
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<tr>
<td>TC</td>
<td>0.060291</td>
<td>0.04513</td>
<td>0.30502</td>
<td>0.20833</td>
<td>0.39774</td>
<td>1.000</td>
<td></td>
</tr>
</tbody>
</table>
The Orientation Index, however, does not specify whether the orientation distributions are statistically random. Thus, the orientation distributions, shown in polar form in Figure 65, were summed for each structure on each substrate, and analyzed for randomness.

Figure 65. F-Actin orientation distributions as determined by 2D FFT for cells grown in (a) 20µm, (b) 30µm, (c) 40µm, (d) 50µm, and (e) 60µm wide microchannels, and on (f) flat PDMS, and (g) TC plastic
Orientation distributions for cells grown on structures where a theoretical mean angle could be determined were analyzed using the $V$ test (Figure 66), while cells grown on flat surfaces were analyzed by the Rayleigh test (Table 13). It is clear from the statistical analysis that the cells grown on microchanneled substrates are not random in orientation, whereas cells grown on flat surfaces are primarily random. However, one data set from the tissue culture substrate showed a rejection of randomness.

![Figure 66. $V$ test values for actin orientation distributions of cells grown on structures with different channel widths](image)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>3-TC</td>
<td>0.004968725</td>
</tr>
</tbody>
</table>
Goodness of Fit. Testing for goodness of fit first required calculation of the concentration parameter, $\hat{k}$, and the mean resultant length, $r$, as shown in Table 14 with the circular standard deviation, $s$. Of the five distributions tested, (von Mises, wrapped normal, wrapped Cauchy, wrapped exponential, and uniform), none provided a satisfactory fit for the data. This is partially due to the background noise found in the data, meaning that all distributions are mixtures containing a uniform component. None of the data sets fit the von Mises distribution well (at a significance level of 0.05 or better); however, it is likely that tests such as the Rayleigh and $V$ tests are still reliable.

Table 14. Values of mean resultant vector length for actin alignment data

<table>
<thead>
<tr>
<th>Structure</th>
<th>$r$</th>
<th>$\hat{k}$</th>
<th>$s$</th>
</tr>
</thead>
<tbody>
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<td>30 µm</td>
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<td>40 µm</td>
<td>0.31417</td>
<td>0.66220</td>
<td>43.59°</td>
</tr>
<tr>
<td>50 µm</td>
<td>0.34576</td>
<td>0.73754</td>
<td>41.75°</td>
</tr>
<tr>
<td>60 µm</td>
<td>0.31434</td>
<td>0.66261</td>
<td>43.58°</td>
</tr>
<tr>
<td>Flat PDMS</td>
<td>0.091884</td>
<td>0.18455</td>
<td>62.60°</td>
</tr>
<tr>
<td>TC plastic</td>
<td>0.12757</td>
<td>0.25726</td>
<td>58.14°</td>
</tr>
</tbody>
</table>

The orientation distributions were then used to acquire angular distributions of alignment as shown in Figure 67. As expected, the two structures with the highest alignment were the two narrowest sets of channels, while the wider channels showed less alignment. The flat surfaces showed the least alignment, with the tissue culture substrates showing only a slight degree of alignment with the mean resultant. Figure 68 demonstrates that although the tissue culture substrates showed a greater alignment than the flat PLL coated substrates, this increase in alignment disappears by 25°, where flat PLL substrates show greater alignment.
Figure 67. Alignment of F-actin filaments with microchannel axes or mean resultant

Figure 68. Cumulative alignment of F-actin filaments with microchannel axes or mean resultant

The mean angle of misalignment is shown in Figure 69. This more clearly demonstrates the alignment inducing ability of the microchannels. The 20 µm wide
microchannel structure shows the best alignment, with the average angle of misalignment being $\Delta \bar{\phi} = 25.8^\circ$. The mean angle increases slightly from 20 µm through 40 µm, where it levels off. The flat substrates show a best case alignment, alignment with the mean resultant, of nearly 45°. A Student’s $t$ test was performed on the calculated means and standard deviations, and the table of $t$ values produced leads to rejection of the null hypothesis that the means are equal for all comparisons of structures with $P$ values below 0.0001 for each. The $t$ values are shown below in Table 15.

![Figure 69. Mean angle of misalignment of F-actin filaments with microchannel axes or mean resultant](image-url)
Table 15. Values from a Student’s $t$ test for comparison of means of misalignment angles of F-actin filaments of cells grown on various substrates. All $t$ values have corresponding $P$ values of less than 0.01 after Bonferroni correction

<table>
<thead>
<tr>
<th></th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
<th>Flat</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 µm</td>
<td>182.7884</td>
<td>308.7436</td>
<td>408.7436</td>
<td>508.7436</td>
<td>608.7436</td>
<td>Flat</td>
</tr>
<tr>
<td>40 µm</td>
<td>582.2416</td>
<td>408.7436</td>
<td>508.7436</td>
<td>608.7436</td>
<td>708.7436</td>
<td>Flat</td>
</tr>
<tr>
<td>50 µm</td>
<td>431.1861</td>
<td>269.9316</td>
<td>106.1433</td>
<td>106.1433</td>
<td>106.1433</td>
<td>Flat</td>
</tr>
<tr>
<td>60 µm</td>
<td>532.8173</td>
<td>376.4317</td>
<td>5.934446</td>
<td>102.5415</td>
<td>102.5415</td>
<td>Flat</td>
</tr>
<tr>
<td>Flat</td>
<td>1179.761</td>
<td>1034.424</td>
<td>665.7321</td>
<td>714.9437</td>
<td>609.0463</td>
<td>Flat</td>
</tr>
<tr>
<td>TC</td>
<td>976.4436</td>
<td>868.0987</td>
<td>591.0058</td>
<td>624.0886</td>
<td>544.6424</td>
<td>75.05225</td>
</tr>
</tbody>
</table>

Analysis of Variance. SAS was used to determine the effect of channel width on the circular variance of actin alignment. The data used to perform the analysis is shown in Table 16. Analysis was also performed to determine if there was a substrate effect on the alignment measurements which would indicate imperfect replication between substrates. The $P$ values obtained show that there is both an effect by the substrate ($P = 0.0035$) and by the channel width ($P < 0.0001$) when testing the hypothesis of equal circular variances.

Table 16. Actin alignment circular variance data for actin alignment

<table>
<thead>
<tr>
<th></th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
<th>Flat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate 1</td>
<td>0.26287</td>
<td>0.309569</td>
<td>0.371645</td>
<td>0.365179</td>
<td>0.391138</td>
<td>0.456642</td>
</tr>
<tr>
<td>Substrate 2</td>
<td>0.267028</td>
<td>0.308093</td>
<td>0.374845</td>
<td>n/a</td>
<td>0.345649</td>
<td>0.450501</td>
</tr>
<tr>
<td>Substrate 3</td>
<td>0.256873</td>
<td>0.256036</td>
<td>0.285789</td>
<td>n/a</td>
<td>0.31811</td>
<td>0.462657</td>
</tr>
<tr>
<td>Substrate 4</td>
<td>0.261182</td>
<td>0.275304</td>
<td>0.325014</td>
<td>0.328225</td>
<td>n/a</td>
<td>0.439531</td>
</tr>
<tr>
<td>Substrate 5</td>
<td>0.216229</td>
<td>0.249225</td>
<td>0.354704</td>
<td>0.293545</td>
<td>0.314563</td>
<td>0.460955</td>
</tr>
</tbody>
</table>

SAS was also used to determine which channel widths produced differences in the actin alignment distribution variances. Table 17 shows the $P$ values for testing of equality of circular variances. All channeled substrates show a considerable difference in circular variance with the flat substrates, underlining the effectiveness of channeled
substrates for aligning cells. Although there is a difference between channels of different widths, the 20 and 30 µm wide channels showed no statistical difference between each other, nor did the 40, 50, and 60 µm wide channels show any difference in actin alignment effectiveness when compared with one another. Basically, the data cluster into three groups: narrow channels, consisting of the 20 and 30 µm wide microchannels, wider channels, comprised of the 40, 50, and 60 µm microchannels, and flat substrates.

<table>
<thead>
<tr>
<th></th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 µm</td>
<td>0.3479</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 µm</td>
<td>&lt; 0.0001</td>
<td>0.0018</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µm</td>
<td>0.0004</td>
<td>0.0181</td>
<td>0.9633</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 µm</td>
<td>&lt; 0.0001</td>
<td>0.0042</td>
<td>1.0000</td>
<td>0.9831</td>
<td></td>
</tr>
<tr>
<td>Flat</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

### Nuclear Alignment

The study on nuclear alignment is critical because it provides a reliable measure of cellular alignment that, unlike the actin fiber alignment experiment, provides a one-sample-per-cell measurement. Orientation angles, $\phi$, were measured over the range, $0^\circ < \phi \leq 180^\circ$, and converted to alignment angles by subtracting the orientation of the microchannels with respect to the horizontal axis of the pictures. This produced an alignment distribution of the nuclei with respect to the microchannel axes as shown in Figure 70. Figure 71 shows the data smoothed by using a histogram with bin widths of $\lambda = 5^\circ$, while Figure 72 shows circular histograms of the data.
Figure 70. Alignment distribution of nuclei with respect to microchannel axes or mean vector

Figure 71. Smoothed alignment distribution of nuclei with respect to microchannel axes or mean vector
Figure 72. Circular histograms of alignment distributions for (II) 20 µm, (IV) 30 µm, (VI) 40 µm, (VIII) 50 µm, (X) 60 µm, (flat) unchanneled, and (rat) in vivo

**Goodness of Fit.** Tests were performed to determine the goodness of fit of various distributions, as certain statistical tests are only applicable for specific distributions. The five distributions tested were the von Mises, wrapped normal, wrapped Cauchy, wrapped exponential, and uniform distributions. To calculate the best fit of each distribution, it was necessary to estimate the concentration parameter, $\kappa$, and the mean
resultant length, \( \rho \), for each population, as shown in Table 18. The alignment distributions of cell nuclei for each structure are shown fit to each of the five distributions tested in Figure 73—Figure 79.

Table 18. Values, \( \hat{k} \), calculated as estimates of concentration parameters, \( \kappa \), from mean resultant vector length, \( r \), and estimator of \( \rho \), by linear interpolation from chart\(^{139}\) for nuclear alignment data with associated circular standard deviations, \( s \)

<table>
<thead>
<tr>
<th>Structure</th>
<th>( r )</th>
<th>( \hat{k} )</th>
<th>( s )</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo</td>
<td>0.8637</td>
<td>4.0094</td>
<td>15.5°</td>
</tr>
<tr>
<td>20 µm</td>
<td>0.7230</td>
<td>2.1649</td>
<td>23.1°</td>
</tr>
<tr>
<td>30 µm</td>
<td>0.6861</td>
<td>1.9297</td>
<td>24.9°</td>
</tr>
<tr>
<td>40 µm</td>
<td>0.5767</td>
<td>1.4239</td>
<td>30.1°</td>
</tr>
<tr>
<td>50 µm</td>
<td>0.5640</td>
<td>1.3764</td>
<td>30.7°</td>
</tr>
<tr>
<td>60 µm</td>
<td>0.5348</td>
<td>1.2732</td>
<td>32.1°</td>
</tr>
<tr>
<td>Flat</td>
<td>0.03906</td>
<td>0.0782</td>
<td>73.0°</td>
</tr>
</tbody>
</table>

Figure 73. Alignment distributions of cell nuclei in 20 µm wide microchannels fit to five probability density functions for circular data
Figure 74. Alignment distributions of cell nuclei in 30 µm wide microchannels fit to five probability density functions for circular data

Figure 75. Alignment distributions of cell nuclei in 40 µm wide microchannels fit to five probability density functions for circular data

Figure 76. Alignment distributions of cell nuclei in 50 µm wide microchannels fit to five probability density functions for circular data
Figure 77. Alignment distributions of cell nuclei in 60 µm wide microchannels fit to five probability density functions for circular data

Figure 78. Alignment distributions of cell nuclei on flat surfaces fit to five probability density functions for circular data

Figure 79. Alignment distributions of in vivo cell nuclei fit to five probability density functions for circular data
For each structure and distribution, a $\chi^2$ goodness of fit test was performed to determine the most appropriate pdf for each distribution. Results of the tests are shown in Table 19 and Table 20. Although the tests do not show any of the distributions to be a good fit, the wrapped Cauchy distribution appears to be best for all structures except for the flat substrate, which is slightly better fit by a wrapped normal distribution.

Table 19. $\chi^2$ value for goodness of fit tests for each structure and pdf

<table>
<thead>
<tr>
<th></th>
<th>in vivo</th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
<th>Flat</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Mises</td>
<td>84.015</td>
<td>306.15</td>
<td>399.72</td>
<td>157.20</td>
<td>195.86</td>
<td>143.84</td>
<td>8.5681</td>
</tr>
<tr>
<td>Wrapped Normal</td>
<td>97.973</td>
<td>421.35</td>
<td>544.70</td>
<td>227.97</td>
<td>277.86</td>
<td>214.91</td>
<td>8.5823</td>
</tr>
<tr>
<td>Wrapped Cauchy</td>
<td>50.589</td>
<td>27.032</td>
<td>19.486</td>
<td>22.583</td>
<td>44.643</td>
<td>20.120</td>
<td>8.5524</td>
</tr>
<tr>
<td>Uniform</td>
<td>1523.5</td>
<td>4915.9</td>
<td>5249.4</td>
<td>1972.1</td>
<td>2364.8</td>
<td>2004.0</td>
<td>11.201</td>
</tr>
</tbody>
</table>

Table 20. $P$ value for goodness of fit tests for each structure and pdf

<table>
<thead>
<tr>
<th></th>
<th>in vivo</th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
<th>Flat</th>
</tr>
</thead>
<tbody>
<tr>
<td>von Mises</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.7393</td>
</tr>
<tr>
<td>Wrapped Normal</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.7381</td>
</tr>
<tr>
<td>Wrapped Cauchy</td>
<td>&lt; 0.0001</td>
<td>0.0076</td>
<td>0.0774</td>
<td>0.0315</td>
<td>&lt; 0.0001</td>
<td>0.0649</td>
<td>0.7406</td>
</tr>
<tr>
<td>Uniform</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.5117</td>
</tr>
</tbody>
</table>

Tests on the axial distributions show that the orientation of cell nuclei is not random, even at the most stringent significance level for available critical values, when cultured on microchannel substrates, as evidenced by the data from a $V$ test shown in Figure 80. Conversely, cells cultured on flat substrates showed no significant alignment, as they were not found to be significantly different from a random distribution, while the $in vivo$ cells showed considerable concentration of orientations, as evidenced by results of the Rayleigh test of uniformity in Table 21.
Figure 80. $V$ test values with critical value, showing rejection of randomness for cells grown on structures with dimensions as given in Table 2

Table 21. $P$ values for Rayleigh test for randomness of distribution of nuclei

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Flat</td>
<td>0.8085967</td>
</tr>
<tr>
<td>3-Flat</td>
<td>0.5042732</td>
</tr>
<tr>
<td>4-Flat</td>
<td>0.5109085</td>
</tr>
<tr>
<td>in vivo</td>
<td>4.85E-124</td>
</tr>
</tbody>
</table>

The alignment distribution is more appropriately displayed as a plot of the angle of misalignment, as shown in Figure 81. These data are more easily visualized as a chart of cumulative percent alignment of nuclei, as shown in Figure 82. These data clearly demonstrate the usefulness of using microchanneled substrates for aligning cells. Cells align with the channels, with this alignment being inversely proportional to the microchannel width. For both the $in vivo$ cells and cells grown on flat PDMS, the microchannel axis was replaced by the mean axis for calculations. Cells grown on flat
surfaces show no preferential alignment with any axis, while cells in native rat tissue show the greatest alignment.

Figure 81. Alignment of cell nuclei with microchannels

Figure 82. Cumulative alignment of nuclei with microchannel axis
The mean and median angles of misalignment are shown in Figure 83. Significance testing by Student’s \( t \) test (Table 22) shows that the mean angle of misalignment (the mean angle between the major axis of the cell nucleus and either the microchannel axis or mean resultant vector if no microchannel is present) is significantly different for all combinations tested except between the 40 µm, 50 µm, and 60 µm wide microchannels with a significance level of at least \( \alpha = 0.01 \). Only the difference of means between the 40 µm and 50 µm microchannels is not significant at any acceptable significance level. While the cells in rat tissue demonstrated the best alignment, most of this increased alignment of \textit{in vivo} cells is within 5° of the mean axis, as is shown above in Figure 81.

![Figure 83. Mean and median angles of misalignment of cell nuclei in various width microchannel substrates](image)
Table 22. *P* values for Student's *t* test for significance of difference in mean angle of nuclear misalignment following Bonferroni correction

<table>
<thead>
<tr>
<th></th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
<th>Flat</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>in vivo</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µm</td>
<td>&lt; 0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 µm</td>
<td>&lt; 0.0001</td>
<td>0.208488</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 µm</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µm</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 µm</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.013893</td>
<td>1.000</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Flat</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

**Analysis of Variance.** To perform an analysis of variance, a SAS code (Appendix B) was used to examine the circular variances and means calculated as described before. The nuclear angular variance was analyzed for effect of channel width and for variations between substrates, or replications of the experiment. The variance data used to perform the analysis are shown below in Table 23.

Table 23. Nuclear alignment circular variance data used for analysis with SAS by substrate and channel width

<table>
<thead>
<tr>
<th>Substrate</th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
<th>Flat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate 1</td>
<td>0.120025</td>
<td>0.140549</td>
<td>0.201268</td>
<td>0.185572</td>
<td>0.233744</td>
<td>n/a</td>
</tr>
<tr>
<td>Substrate 2</td>
<td>0.111432</td>
<td>0.156182</td>
<td>0.215174</td>
<td>0.247597</td>
<td>0.249211</td>
<td>0.488828</td>
</tr>
<tr>
<td>Substrate 3</td>
<td>0.159605</td>
<td>0.190952</td>
<td>0.229576</td>
<td>0.200737</td>
<td>0.226694</td>
<td>0.475349</td>
</tr>
<tr>
<td>Substrate 4</td>
<td>0.151594</td>
<td>0.155321</td>
<td>0.204336</td>
<td>0.236087</td>
<td>0.226254</td>
<td>0.465452</td>
</tr>
</tbody>
</table>

Analysis of the data show that the substrate has very little effect, if any, on circular variance of nuclear alignment (*P* = 0.1980) while the channel width has a large effect (*P* < 0.0001) for testing equality of circular variances. Further analysis on the data shows that the circular variances cluster in the same three distinct groups as did the actin data (20 µm to 30 µm, 40 µm to 60 µm, and flat), as demonstrated by the results in Table
The nuclei of cells cultured on the flat control surface are found to have a circular variance that is significantly different than the nuclei of cells grown on any of the channeled substrates. Also, the two narrowest channels, 20 and 30 µm widths, show a significantly larger ability to align nuclei than the wider channels of 40, 50, and 60 µm, as the circular variances between these two distinct groups are significantly different between groups but not within. All channel widths show better alignment than flat surfaces.

Table 24. *P* values for null hypothesis that nuclear circular variances are equal, using Tukey-Kramer adjustment for multiple comparisons

<table>
<thead>
<tr>
<th></th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 µm</td>
<td>0.3479</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 µm</td>
<td>&lt; 0.0001</td>
<td>0.0018</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µm</td>
<td>0.0004</td>
<td>0.0181</td>
<td>0.9633</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 µm</td>
<td>&lt; 0.0001</td>
<td>0.0042</td>
<td>1.0000</td>
<td>0.9831</td>
<td></td>
</tr>
<tr>
<td>Flat</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

**Nuclear Roundedness**

The nuclear roundedness data provide a measure of how circular the nuclei are in a plane parallel to the cell culture growth surface. Higher values of roundedness indicate more circular nuclei, whereas low values of roundedness indicate more elliptical nuclei. As cells become more elongated, they may constrict the shape of the nuclei, thus nuclear roundedness can be a measure of cell elongation. The smoothed data presented below in Figure 84 show that cells from rat tissue have a much higher roundedness than that found in cells grown on cell culture scaffolds. The mean value for percent roundedness is shown in Figure 85.
Figure 84. Percent roundedness of nuclei of cells grown on structures of different channel widths

Figure 85. Mean percent nuclear roundedness by structure
There appears to be a general trend towards more rounded cells as the channel width is increased. A Student’s $t$ test was performed to test the significance of the difference in means. The $P$ values from this test are found in Table 25. These data show that there is a real difference in nuclear roundedness between cells grown in channels of different widths. Although not all the differences are significant at high confidence levels, it is clear that the trend is real and holds over all structure widths.

<table>
<thead>
<tr>
<th></th>
<th>in vivo</th>
<th>20 µm</th>
<th>30 µm</th>
<th>40 µm</th>
<th>50 µm</th>
<th>60 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 µm</td>
<td>&lt; 0.0001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 µm</td>
<td>&lt; 0.0001</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 µm</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µm</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>2.1649E-04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 µm</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>0.95833</td>
<td></td>
</tr>
<tr>
<td>flat</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Table 25. $P$ values for Student's $t$ test for comparison of mean roundedness between structures.
CHAPTER 4

DISCUSSION

The results presented in this thesis point to the overall conclusion that microfabricated microchannels are extremely effective in inducing cellular alignment. This, however, is only one of many parameters affecting cellular and tissue function, so the structures used to measure the effect of alignment on cells would need to be modified to alter other aspects of cellular behavior. The cell properties discussed here are nuclear and filamentous actin orientations, actin and myosin densities, cell density, cell viability, and nuclear roundedness. The ramifications of these data will be discussed in turn, following a discussion of the substrate metrology data. The limitations of the methods used for measuring the quantities reported, and how these may affect the statistical significance of the data, will also be discussed.

Scaffold Metrology

The results from the scaffold metrology show that the PDMS scaffolds are nearly perfect replicates of the silicon master, and they are close to the designed values for depth and width. Three different types of metrology, each with limitations, were used to analyze the physical structure of the substrates and the silicon wafer mold: RST, surface profiler, and SEM. Further analysis was performed to determine the degree of uniformity
of the PLL deposition upon the PDMS substrates, with emphasis on the layer thickness as a function of channel width.

**Surface Profiler Metrology**

The surface profiler metrology proved inappropriate for measuring the depths and widths of the microchannels, because either the stylus did not enter the channels (due to their high aspect ratio), or the PDMS was not structurally rigid enough to withstand the pressure, and was flattened by the profiler. As a result, the measured channel depths are considerably smaller (~6 µm) than those found by either SEM image analysis (~12 µm) or RST analysis (~17 µm). The widths (measured at the bottoms of the channels) could not be calculated either, since the profiles did not adequately display the edges of the microchannel walls (demonstrated by Figure 44). For this reason, it appears that the data obtained from the surface profiler are not sufficiently reliable for measuring the depth and widths of channels.

**RST Metrology**

The RST metrology showed results more closely matching that from the SEM analysis. However, it was difficult to accurately obtain measurements for depth from the PDMS microchanneled substrates due to their optical transparency and low reflectance. Sputter coating with palladium did not significantly lower the variance of the channel depths, though more measurements may have corrected this problem.

**SEM Metrology**

Images taken of the PDMS scaffolds viewed under an SEM clearly show the profiles of the channels in cross section. Values obtained for widths and depths show a low variance and agree with the expected means, as shown in Figure 47. The
microchannels have a mean deviation of 3.4% from the designed channel width, and a maximum depth only 7.7% greater than the minimum depth. As expected, these data clearly illustrate the reliability of ICP etching for production of detailed microstructures, and the utility of the PDMS molding process to replicate these features from a silicon master.

**Analysis of PLL Coverage**

The method for measuring the PLL coverage was not adequate for quantifying the depth of the PLL layer deposited; however, it clearly demonstrated that PLL was indeed layered on the PDMS substrate when preceded by the polyelectrolyte film used, and did not assemble on plain PDMS. A more quantitative method would have been to use a fluorophore less susceptible to photobleaching, such as Alexa Fluor 488. This would have eliminated the problem introduced by the fading of the fluorophore. Another problem was the camera itself. A black and white camera should have been used for measuring the fluorescence light intensity resulting from the excited fluorophore to provide a more accurate measure of the light emitted. These two improvements would have yielded more reliable results from PLL-associated fluorescence intensity. Despite these problems, the procedure used does conclusively show that the PLL adhesion is uniform and is dependent on the precursor layers of PEI, PSS, and PDDA.

**Cellular Density and Viability**

Cell density was calculated by two different methods, a manual count and an automated count utilizing a blob analysis image processing method. The density was calculated by dividing the number of cells per image by the image area. The two
methods used to enumerate cells differed in their final cell density estimates for a variety of reasons. The most significant difference results from the magnification in the images used. For more accurate cell counts, lower magnification images with larger field areas were preferred. The images taken with the 10x objective were analyzed by manually counting nuclei; however, the pictures did not have sufficient resolution to permit automated counting because nuclei in a close proximity to one another were not likely to be resolved as individual cells. Regardless, cells in the 100x magnification images were more difficult to count and more likely to yield an error in counting than if the 400x magnification images were manually counted.

Some of the images used for the blob analysis were also counted manually to determine the ability of the automated method to accurately assess cell density. The two methods, discussed earlier, resulted in counts differing by 7.15%. The difference between the two methods was not random, however, with automated counts consistently undercounting the cell nuclei. There are two primary reasons for this situation. First, the nuclei are not randomly distributed throughout the surface of the substrate, but often occur in pairs as the cells undergo division. Often, two closely placed cell nuclei are included in one blob during the analysis. As a result, highly proliferative cultures may incur a substantial undercount. However, since in most cases the cells that occur in pairs are cells that have recently divided, it is perhaps not a significant error with the undercount being fairly representative of the cell population at a time not too long previous to when the measurement was taken. Furthermore, the analysis is relative, so the ratio of cell densities between structures is more important than the density itself.
A second cause for error in cell counting can be traced to difficulties in accurately thresholding the images to produce a two-bit image readable by the MATLAB script. As a result, nonuniform fluorescence excitation caused some cells near the edges of the image to appear darker and thus occasionally below the threshold used. The cells were not counted by the automated method but were perhaps counted by the manual method, thus introducing more inconsistency between the two counting methods.

Despite all the differences between the two counts, they affected all cell cultures uniformly, thus producing no real difference for the measured ratio of actin and myosin densities between cells grown on the various structures. The dead cell counts were of lesser reliability. Although the RNase A significantly reduced the cytoplasmic staining associated with the ethidium homodimer-1 binding with RNA instead of DNA, it significantly complicated manual counting of dead cell nuclei from 100x magnification images. Thresholding significantly aided in discrimination of cell nuclei from cytoplasm for the 400x magnification images allowing for reliable cell counts to be obtained. For this reason, the 400x magnification automated counts are preferred for cell viability estimates.

**Cell Density**

The measurements for cell density indicate an increase in cell density of approximately 300% in six days. Variations in cell density between different structures are likely due not to a difference in rates of cell proliferation between cells in different structures, but rather a nonuniform cell seeding density. Though not quantified, cells were found to become more dense toward the middle of a well immediately upon seeding. This was not alleviated even by thorough mixing of cells prior to addition of
cells to the wells, and cells were not easily mixed after adding cells, as they quickly sank to the bottom and into the channels from which they could not be removed to create a more uniform distribution. Because no initial cell count was obtained immediately after seeding, no conclusion can be drawn about the ability of the microchannels to affect cell proliferation rates by varying the width of the microchannels.

The values for cell density are crucial for several other measurements, however, such as actin density and myosin density. Also, both total cell density, measured as the density of Hoechst 33342-stained nuclei, and dead cell density, the density of nuclei stained with ethidium homodimer-1, are required for measurements of cell viability.

Cell Viability

Cell viability could potentially be affected by both microchannel width and the surface chemistry. Both a narrow channel width and the appropriate surface charge can increase cell adhesion, thus promoting a higher viability. Conversely, deep, narrow channels can potentially decrease the viability of cells due to the limitation of diffusion. Since viability was shown to not decrease with decreased channel width, it can be proven conclusively, though not surprisingly, that for channels of the depths used (10-15 µm), the 20 µm channel widths were not narrow enough to have a significant impact on diffusion of nutrients and waste products.

In order to adequately address the effect of surface chemistry on the ability of cells to proliferate, it would be necessary to culture cells on substrates with a wide variety of surface chemistries, and that is outside the scope of this research. However, since the surface chemistry is identical for all substrates, any actual change in viability with channel width should be an effect of the channel geometry. Further tests would need to
be run to see if this increase in viability experienced by cells grown in microchannels is
due to better adhesion to the channeled substrates or some other unknown effect.

Cellular Alignment

Two different indicators of cellular alignment were analyzed to determine the
ability of the microchanneled scaffolds to align cells: nuclear orientation and actin
filament orientation. The nuclear orientations were analyzed by a custom blob analysis
script written for MATLAB that measured the orientation of individual nuclei. In contrast,
the actin filament orientation distributions were obtained by performing a two-
dimensional Fourier transform on fluorescence images of cells stained with phalloidin.
The methods of analysis were selected based on the features of the objects to be analyzed.
The use of two different analysis methods accounts for some of the discrepancy between
the two alignment indicators; however, there are other factors involved. Most
importantly, while each nucleus is aligned, more or less, with the long axis of the cell,
actin filament orientations show some variability within the cell. Despite this, the
significance testing of the circular variance of the actin and nuclear alignment
distributions show that data from both measurements cluster into the same three distinct
groups: 20 and 30 µm wide channels, 40 to 60 µm channels, and flat substrates.

Actin Filament Alignment

The actin filaments show a clear trend of greater orientation with decreasing
channel width. Despite this, it is not a perfect relationship as the data indicate that 50 µm
wide microchannels ostensibly promote greater alignment than 40 µm wide
microchannels. While perhaps the most likely explanation for this is that the difference
between the two channel widths is not significant, thus allowing natural random variations to cause the apparent contradiction with the theory of cellular alignment in microchannels, there are other possible reasons for this phenomenon. Since the actin filaments extend toward areas where the cells form attachments, more dense cultures may show more deviation from alignment as cells seek to attach to adjacent cells. Also, as the channels become narrower, it may cause cells to be more likely to attach with each end attached to a microchannel wall, crossing the channel diagonally. Overall, there is a clear indication that alignment is induced by narrow microchannels.

**Nuclear Alignment**

Nuclear alignment is perhaps the best indication of cell alignment, since there is only one nucleus per cell, whereas actin filaments present a distribution of orientations for each cell. Also, while the measurement of orientation distributions for actin filaments is not particularly straightforward, each data sample for nuclear orientations is a measurement of the orientation of a particular and identifiable nucleus. Although at first it may appear that the blob analysis method used for nuclear orientations is better than the 2D FFT used for the actin filament orientations, there are certain problems encountered with overlapping nuclei. The 2D FFT is not adversely affected by overlapping filaments. However, the blob analysis method fails for overlapping cells, since the resulting principle axis for two conjoined cells is not necessarily related to the principle axis of either of its component cells. This problem can be remedied by removing multi-cell blobs from analysis by setting an upper limit (in terms of pixel size) for areas of blobs to be analyzed.
The results of the nuclear alignment distributions show a strong agreement with the hypothesis that narrow channels will promote increased alignment. Unlike the actin filament data, which show some inconsistencies, the nuclear orientation data show a consistent trend across all channel widths. Even though analysis of variance showed no significant difference in the alignment for every width comparison, the narrower channels (20 and 30 µm) were significantly better at aligning cell nuclei than the wider channels (40, 50, or 60 µm), which were also better than the flat control substrates. Also, cells from *ex vivo* tissue samples show alignment of cells that is greater than that of the 20 µm wide channels. From these data it is known what a normal orientation distribution of nuclei in smooth muscle cells is in rat aortas. It has also been shown that the 20 µm wide microchannels are insufficient to produce the degree of alignment found in cell from *ex vivo* tissue samples. Since the *in vivo* nuclear orientation distribution is outside the range measured for cells grown on channels with widths between 20 and 60 µm, no attempt was made to calculate the channel width that would most likely replicate the orientation distribution found *in vivo*. Although microchannels alone with 20 µm widths did not produce a degree of alignment equal to that observed *in vivo*, other methods for inducing alignment, such as pulsatile flow of cell culture media, cyclical stretching of the cell culture substrate, or contact guidance by microgrooves, could by used with microchannels to produce the desired alignment of cells.

**Cell Phenotype Characterization**

Two measurements intended to characterize the cell phenotype were taken, quantifying actin and myosin density. Both analyses were inconclusive due to the
reasons outlined below. The two underlying factors that led to the poor results were the cell seeding methods and the image acquisition. First, the cells did not seed uniformly throughout the substrate, rather they settled more densely in the center of the scaffold no matter how well mixed the cell suspension was. The cells settled quickly into the channels from where they were not easily moved to produce a more uniformly dense cell population. This alone should not have been a major problem except that cell phenotype was hypothesized to be influenced by cell density. As a result, there are two variables introduced, channel width and cell density.

The second contributing factor to the inconsistent results was the camera used to obtain images. A superior camera that was more capable of reliably measuring light intensities would have allowed for more dependable results from the image analysis. This would have been most useful for the actin density measurements where it was necessary that the cells remain in place so that accurate orientation distribution calculations could be made. For the myosin density, it would have been preferable to remove the cells and do an ELISA (Enzyme Linked Immunosorbant Assay) to quantify the amount of myosin, using cells trypsinized and removed from the structures, with the optical density measured by a plate reader designed for that purpose rather than a digital camera. However, removing cells from the PDMS microchannel substrates layered with polyelectrolytes is difficult, partly due to the charge of the substrate, and its attraction of the cells, but also due to the structure, and the complication of removing the cells that have settled in narrow, deep microchannels.

Also, a large number of cells would have been required to perform an ELISA, thus making ELISA, although an attractive technique, not appropriate given the
constraints imposed. The small number of substrates used, which was a result of the time available to complete the study, limited the number of cells and, thus, measurements taken. The number of substrates, the restricted area of each structure on each substrate, and the natural variability of cells all contributed to a very high variability in the measurements that shrouded any effect that could have been observed.

With all the complicating factors listed above, it is not surprising that any effect of microchannel width on cell phenotype (as characterized by myosin and actin density) were not noticeable. The studies do not demonstrate anything other than that the cells possess a level of actin and myosin that is not excessively different from cells grown on unstructured surfaces. Future work on the methodology for phenotype characterization could possibly prove whether or not there is an observable difference in cell phenotype for cultures grown in microchannels as opposed to those grown on standard flat surface substrates.

**Actin Density**

The results for actin density, as determined by analysis of fluorescence intensity associated with Alexa Fluor 488-phalloidin bound to F-actin filaments, show no variation in actin density associated with channel width. Although variations do exist between the various channel widths, it cannot be ruled out that it is a result of an inaccurate cell density measurement, since more variation is displayed in the actin density than total actin content measured.

**Myosin Density**

The myosin density does show a possible trend towards greater myosin content in narrower channels; however, the low number of samples and the large variance in the
data do not allow any specific statistically significant conclusions to be drawn concerning whether channeled substrates promote a change in cellular differentiation as measured by myosin content.

**Nuclear Roundedness**

The nuclear roundedness measurements are used to quantify cell morphology. Normal, elongated cells generally have elongated nuclei due to the geometric constriction of the cell. As a result, nuclear elongation, or its converse, nuclear roundedness, can be used to infer the geometric shape of the cells. This measurement was taken by considering the ratio of the lesser to the greater of the principal axes of the cell nuclei, as measured by blob analysis from digital fluorescence micrographs as described previously, and expressed as a percent roundedness.

The measurements obtained show a clear trend: as the microchannels become narrower, the cells are more highly elongated, and have a lower value for percent nuclear roundedness. Also, cells from tissue samples show a much lower value for nuclear roundedness than do cells grown on the PDMS scaffolds. However, these cells cannot be directly compared with one another without considering the complicating effect of two-dimensional and three-dimensional cultures. Since the cells grown on the PDMS scaffolds were cultured on flat surfaces, this may effect how the cells elongate, because cells may become wider when grown on flat surfaces to attach securely to the surface. As a result, the higher values for nuclear roundedness on the structures may be due to being cultured on flat surfaces.
Future Work

A wide variety of modifications could be made in future work. The substrates could be modified to have wider and narrower channels to provide a wider range in the effect induced by the microchannels. This would be more likely to demonstrate a statistically significant difference in cell density and contractile protein production. Also, the structures of different channel widths should be separated so that the seeding density can be more carefully controlled. In addition, different ECM components could be applied to the substrate as well as the addition of endothelial cells or pulsatile flow. These would be more likely to reproduce the in vivo structure of an artery.

Different types of analysis could be performed for other markers of contractile smooth muscle cells, such as smoothelin, which has been shown to be a suitable marker for contractile cells. Measurement of extracellular matrix components such as collagen and elastin would also be beneficial, since these are essential for normal function of arteries. Alternate methods for measuring the desired quantities would also improve the resulting data since the analysis of the cellular phenotype data suffered from the quality of data. Lastly, a quantitative method for determining cell contractility is necessary to ensure that the cells are functional.

Conclusions

The ability of microchannel cell culture scaffolds to align smooth muscle cells has been demonstrated by two methods analyzing separate characteristics of cell alignment, the orientation distributions of actin filaments and nuclei. The goal of producing a cell culture scaffold capable of aligning smooth muscle cells into a highly dense culture was
achieved, although no significant effect on cell phenotype was observed. Furthermore, cell nuclei were found to become elongated when cells were cultured in microchannels. Both elongation and alignment were found to be greater in narrower channels. Consequently, microchannel cell culture scaffolds can be shown to be valuable for research in tissue engineering.

Microchannel cell scaffolds with 20 to 60 µm channel widths separated by 10 µm wide walls were accurately replicated from etched silicon masters using PDMS micromolding. Surface modification of the elastomer substrates were performed using layer by layer assembly of polyelectrolytes, including PLL, which was observed to be uniform and independent of channel width.

The investigation into the effects of microchannel cell culture substrates on cellular phenotypic expression using actin and myosin density measurements, produced data that is inconclusive. Neither the analysis of the actin density nor the myosin density data produced results indicative of an effect on cell function and proliferation by the microchannels. This is partly due to a wide variety of factors, such as the limited culture time, the small sample sizes, as well as the nonuniform cell seeding. For this reason, it is not possible to conclude that microchannel cell culture scaffolds has any effect on the cellular content of either of these two proteins.

The studies into cellular alignment were more significant, yielding data that clearly show the effect of microchannel cell culture substrates on the orientation of cells. Furthermore, it is evident that the width of the microchannels is an important factor in determining the degree of cellular alignment. Despite this increased alignment by
microchannel cell culture scaffolds, a degree of alignment comparable to that found in *in vivo* tissue is not produced by the range of microchannel widths tested.

The actin filament analysis by 2D FFT demonstrated that actin filaments preferentially align with the axis of the microchannels, and that this effect was dependent on channel width. Narrow channels have cells with highly aligned actin filaments, while wider channels have less highly aligned actin filaments. Flat surfaces have cells with actin filaments that are statistically random in most instances. Nonrandom alignments on flat surfaces may be due in part to the size of the picture area used for the image analysis, potentially indicating some self-induced alignment over small areas. The experiments performed on actin filament alignment indicate a clear trend favoring orientation of F-actin filaments in the direction of the microchannel axis. This is critical for the development of functional smooth muscle tissue.

Like the actin alignment experiment, the results from the nuclear alignment experiment prove that nuclei are aligned by microchannels, with narrower channels producing a greater effect of alignment than wider channels. Flat surfaces, like those commonly used for culturing cells, produced statistically random orientations for cell nuclei. Cells from native rat tissue were also stained for nucleic acids to provide data for nuclear alignment. The data show that cells *in vivo* are highly aligned, unlike that found in culture on standard tissue culture surfaces. Cells cultured in microchannels with widths of 20 µm were found to have mean angles of misalignment approaching, but not equal, that of native rat tissue. Thus, to provide alignment equivalent to that found *in vivo*, it will be necessary to use narrower channels or add other factors known to promote cell alignment.
Nuclear roundedness data also confirms that microchannel tissue culture substrates produce cells with traits similar to *in vivo* cells. Namely, the nuclei, and thus the cells themselves, have a more elongated morphology when the cells are cultured on microchanneled cell culture substrates. This increase in elongation is shown to follow a decrease in microchannel width. Since it is known that contractile cells have a more elongated morphology than those typically found cultured on flat surfaces, it is a significant finding that cells are not just more highly aligned, but also more elongated when cultured in microchanneled substrates.

Although there is no conclusive evidence that demonstrates a phenotypic difference between cells cultured on microchannels and on flat surfaces, it has been shown that there are observable effects resulting from the microchannels on the cell culture. The data clearly demonstrate that cells cultured on microchannel cell culture substrates show morphology closer to that of *in vivo* cells than do those cultured on flat cell culture substrates in both the degree of alignment and elongation. This effect is inversely proportional to the microchannel width. These results show that microfabricated high aspect ratio microchannels provide a highly effective substrate for the culture of aligned smooth muscle cell cultures. In the future, experiments can be performed to determine if the altered morphology produced induces a resulting change in cell phenotype. This would indicate that the cell phenotype can be controlled by cell morphology and would provide valuable information for future tissue engineering efforts.
APPENDIX A

MATLAB SCRIPTS

Two-Dimensional Fourier Transform Image Analysis Script

% John D. Glawe
% autoTwoDfftver2.m
%
% Input of program is a set of grayscale, 2-dimensional (non-RGB) pictures
% Images should be squares, and are padded to 2^N (N=integer) increase resolution (1024+)
% Prior to padding, a Gaussian filter window is applied to smooth edges and remove
% horizontal and vertical frequency components due to edge effects.
% After the 2D FFT is taken, images are bandpassed with user specified low and high cutoff
% frequencies. These are in terms of cycles/image. A low cutoff frequency (inner radius)
% of 57 creates a circumference equal to 360, providing (approximately) one degree resolution.
% The high cutoff frequency (outer radius) is expressed in terms of multiples of the low cutoff
% frequency. A value of 1.25 works well for 1024 x 1024 images.
% The 2D FFT power spectrum, after bandpassing, is converted to cylindrical coordinates and an
% orientation distribution is created that corresponds to magnitudes from the 2D FFT power
% spectrum at right angles to the lines in the image. The orientation distribution gives the
% intensities of lines at various angles in the image, with 0 degrees being horizontal, and
% angles increasing counterclockwise. Data is exported to a user named
% file in comma separated values (.csv) format
%
% VARIABLES (EXCLUDING DUMMY VARIABLES)
% %
% LOWFREQ    low pass frequency for 2D FFT spectrum with units of cycles per image width (This is FINAL padded width)
% HIGHCUTFACTOR equals highpass frequency divided by lowpass frequency
% TOTALLOOPS number of images analyzed
% DATAFILENAME user specified output file in .csv format
% PADLENGTH side width to which to pad image
% INTENSITYDATA contains matrix of angles and magnitudes at those angles
% PICTURE Two dimensional image (non-RGB) analyzed in current loop
% G Gaussian filter window equation (see reference)
% FOURIER Two dimensional Fourier transform of image in current loop

clear

titlename = 'John Glawe''s Automatic Orientation Analyzer';

question1 = {'Enter low cutoff frequency (cycles/image) for Fourier bandpass. (Enter 57 for minimum that preserves one degree resolution.)'};
lowfreq = str2num(answer1{1,1});

answer2 = inputdlg('Enter ratio of high cutoff frequency to low cutoff frequency.',titlename,1,{'1.25'});
highcutfactor = str2num(answer2{1,1});

answer3 = inputdlg('Enter number of samples to analyze.',titlename,1,{'1'});
totalloops = str2num(answer3{1,1});

answer4 = inputdlg('Enter output filename. Data will be in .csv format.',titlename,1);
datafilename = answer4{1,1};

answer5 = inputdlg('Enter size to which to pad image (must be 2^N)',titlename,1,{'1024'});
padlength = str2num(answer5{1,1});

intensitydata = zeros(180,totalloops);

% Loop to get file names
for fileloop = 1:totalloops;
clear filename pathname
boxtitle=['Select Grayscale Image File ' num2str(fileloop) ' of ' num2str(totalloops)];
[filename(1,:),pathname(1,:)]=uigetfile('*.tif',boxtitle);
filenames{fileloop}=filename;
fullpath=[pathname,filename];
fullpaths{fileloop} = {fullpath};
end

tic
% Loop to perform Fourier analysis
clear picture g padpicture fourier fourierscaled theta rho z
thetavect zvect intensity
for analysisloop = 1:totalloops;
    % Get image
    picturename = fullpaths{analysisloop};
    picture = double(imread(picturename));
    n = size(picture);
    N = n(1); % Get width of picture

    % Gaussian Filter Window
    for x = 1:N;
        for y = 1:N;
            % Below formula adapted from Palmer and Bizios, Journal of Biomechanical Engineering, vol. 119, 1997, p.160 where N is
            % mult. by 0.396*N
            g(y,x) = exp(-((x-N/2)^2 + (y-N/2)^2)^2/(N^4/100));
        end
    end
    picture = picture.*g;
    picture = picture - mean(mean(picture)); % Remove DC

    % Add zero padding to improve resolution
    if padlength>N;
        padpicture = zeros(padlength,padlength);
        padstart = padlength/2-N/2+1;
        padpicture(padstart:(padlength-padstart+1),padstart:(padlength-padstart+1)) = picture;
        picture = padpicture;
        N = padlength;
    end

    fourier = (abs(fftshift(fft2(picture)))).^2;

    % Convert Cartesian coordinates to Cylindrical coordinates
    for x = 1:N;
        for y = 1:N;
            theta(y,x) = atan((y-N/2-1)/(x-N/2-1+.0001*(x==(N/2+1)))); % Addition of .0001 necessary to prevent division by zero
            rho(y,x) = sqrt((y-N/2-1)^2+(x-N/2-1)^2); % Bandpass filter
            z(y,x) = fourier(y,x)*(rho(y,x)<lowfreq*highcutfactor)*(rho(y,x)>lowfreq);
            % Bandpass filter
        end
    end

    % Convert theta matrix to degrees
    % Flip theta first because of flipped y axis in Matlab
    % Shift 90 degrees because spectrum is of waves perpendicular to filaments
    thetavect(1:N^2) = ((flipud(theta))*360/2/pi)+90;
% Round theta vector to whole numbers
thetavect = round(thetavect);

% Shift data from theta = 0 to theta = 180 to eliminate 0 matrix index
thetavect = thetavect +180.*(thetavect==0);

% Transfer z data (intensity) from z matrix to z vector
zvect(1:N^2)=z;

% Initialize intensity vector
intensity = zeros(1,180);

% intensity is a vector of 180 elements whose value corresponds to the intensity at that angle
% thetavect is a vector of N^2 elements whose value corresponds to the angle of that pixel
% zvect is a vector of N^2 elements whose value corresponds to the intensity of that pixel
% Sum all z's for a particular theta and move to intensity vector
for loop = 1:N^2;
    intensity(thetavect(loop)) = intensity(thetavect(loop)) + zvect(loop);
end
intensitydata(:,analysisloop) = intensity';

highfreq = highcutfactor*lowfreq;

% Loop to write data to file
datatags = (1:180)';
nametags(1,1) = {'filename >'};
nametags(1,2:totalloops+1) = filenames';
 fid=fopen(datafilename, 'w');
 for loop = 1:totalloops+1;
    fprintf(fid, '%s,',char(nametags(loop)));
 end
 fprintf(fid, '
');
 for loop = 1:180;
    fprintf(fid, '%3.0f,',datatags(loop));
    for loop2 = 1:totalloops;
       fprintf(fid,'%4.4f,',intensitydata(loop,loop2));
    end
    fprintf(fid,'
');
 end
 fprintf(fid,'
%s,','Picture size');
 fprintf(fid,'%4.0f,',picsize);
 fprintf(fid,'
%s,','low cutoff freq.');//fprintf(fid,'%4.3f,','lowfreq);
```matlab
fprintf(fid,\"\n%s,\",'high cutoff freq.');
fprintf(fid,\'%4.3f,\',highfreq);
fclose(fid);
toc
disp('Processing finished.')
disp([\'Data in file: \',datafilename])
```

### Automatic RGB Color Intensity Analyzer

1 % John Glawe
2 % Louisiana Tech University
3 % Institute for Micromanufacturing
4 %
5 % This program measures the average red, green, and blue
6 % intensities
7 % of RGB images supplied by the user as well as color
8 % saturation.
9 %
10 clear
11 % Request pictures from user
12 title = 'John Glawe''s RGB Intensity Analyzer';
13 answer = inputdlg('How many files to quantify?',title,1);
14 numberoffiles=str2num(answer{1,1});
15 answer = inputdlg('Enter output filename. Data will be in .csv
format.',title,1);
16 datafilename = answer{1,1};
17 datafilename = answer{1,1};
18 for fileloop = 1:numberoffiles;
19     clear filename pathname
20     boxtitle=['Select JPEG Image File ' num2str(fileloop) ' of ' num2str(numberoffiles)];
21     [filename(1,:),pathname(1,:)]=uigetfile('*.jpg',boxtitle);
22     filenames(fileloop)=filename;
23     fullpath=[pathname,filename];
24     fullpaths(fileloop) = {fullpath};
25 end
26 % Analysis loop
27 tic
28 for fileloop = 1:numberoffiles;
29     % Get image
30     picturename = fullpaths(fileloop);
31     picture = double(imread(picturename,'jpg'));
32     [row,col,colors]=size(picture);
33     pixels=row*col;
34     red=zeros(pixels,1);
35     green=red;
36     blue=red;
37     red = picture(1:pixels);
38     green = picture(pixels+1:pixels*2);
39     blue = picture(2*pixels+1:pixels*3);
```
rgb(:,1) = red';
rgb(:,2) = green';
rgb(:,3) = blue';
sat(fileloop) = mean(max(rgb)-min(rgb))/255;
redavg(fileloop) = mean(red);
greenavg(fileloop) = mean(green);
blueavg(fileloop) = mean(blue);
end
% Save data to file
fid=fopen(datafilename, 'w');
fprintf(fid,'Filename,Avg Percent Saturation,Avg Red Intensity,Avg Green Intensity,Avg Blue Intensity,
');
for loop=1:numberoffiles
  savedata = [sat(loop), redavg(loop), greenavg(loop), blueavg(loop)];
  fprintf(fid, '%s,%4.3f,%4.3f,%4.3f,%4.3f\n',char(filenames(loop)),savedata');
end
fclose(fid);
toc
disp('Processing finished.')

Automatic Blob Analyzer

% THESISBLOBANALYZER.m
% John D. Glawe
% Created 08/18/2002
% Modified 08/29/2003

clear

titlename = 'John Glawe''s Blob Analyzer';
edgeelim = questdlg('Eliminate edge objects? (Recommended)',titlename);

questions = {'Enter minimum blob size to analyze','Enter maximum blob size to analyze'};
answer = inputdlg(questions,titlename,1,'0','0');
minblobsize = str2num(answer{1,1});
maxblobsize = str2num(answer{2,1});

answerthresh = inputdlg('Select threshold intensity.',titlename);
thresh = str2num(answerthresh{1});

% JPG files will be read from folder: D:\Students\John\matlab\blob'
Based on names of images, determine where Thumbs.db will appear (in alphabetical order) and adjust removal of Thumbs.db appropriately. Also adjust size of Thumbs.db by space padding
directory = 'D:\Students\John\matlab\blob'\;}
answer3 = inputdlg('Enter output filename. Data will be in .csv format.',tifilename,1);
datafilename = answer3{1,1};
tic

% Get file names
folder = dir(directory);
[numberoffiles,x] = size(folder);
filenames = struct2cell(folder);iles = filenames(1,3:numberoffiles);
filenames = char(files);
numberoffiles=numberoffiles-2;
if filenames(numberoffiles,:) == 'Thumbs.db
  numberoffiles = numberoffiles-1;
end

% Analysis loop
for fileloop = 1:numberoffiles;
  % Get image
  fullpath = [directory,filenames(fileloop,:)];
picture = imread(fullpath,'jpg');
[y,x,z] = size(picture);
pixels=x*y;

  % Intensity image
  grayscale=double(picture);

  % Optionally insert automated thresholding routine here
  grayscale(:,:,1)=(grayscale(:,:,1)>thresh);

  % Begin Blob Analysis Loops
  yblob=zeros(y,x);
yblobnum=1;

  % Search pixels vertically for blobs
  for yblobloop = 2:pixels;
    % Increase blob number if current pixel value larger than previous pixel
    yblobnum = yblobnum + (grayscale(yblobloop)>grayyscale(yblobloop-1));
    % yblob = matrix of values of blob numbers that each pixel is a part of
    yblob(yblobloop) = yblobnum*grayyscale(yblobloop);
  end

  % Consolidate blobs horizontally
  for loop = 1:pixels-y;
    if yblob(loop)*yblob(loop+y) > 0;
      if yblob(loop) == yblob(loop+y)
        ymin=min(yblob(loop),yblob(loop+y));
ymax=max(yblob(loop),yblob(loop+y));
yblob(:,:,1)=yblob(:,:,1)-((ymax-ymin)*(yblob(:,:,1)
          ==ymax));
      end
    end
end
maxloops = max(max(yblob));

% Remove blobs smaller and larger than user set values
for loop = 1:maxloops;
    yblob = yblob - (sum(sum(yblob==loop))<=minblobsize).*yblob.*yblob==loop);
    yblob = yblob - (sum(sum(yblob==loop)>=maxblobsize).*yblob.*yblob==loop);
end

if edgeelim(1)=='Y'
    % Remove edge blobs and blobs that connect by wrap from bottom to top
    for loopy = 1:y;
        if yblob(loopy,1)+yblob(loopy,x-1)~=0
            yblob = yblob.*(yblob~=yblob(loopy,1)) .*(yblob~=
        yblob(loopy,x-1));
    end
    for loopx = 1:x;
        if yblob(1,loopx)+yblob(y,loopx)~=0
            yblob = yblob.*(yblob~=yblob(1,loopx)).* (yblob~=
        yblob(y,loopx));
    end
end

% Set blob 0 (background) above highest blob
yblob(:,:,)=yblob(:,:,)+(maxloops+1)*(yblob(:,:,)==0);
blobnumber = 1;

% Renumber blob that has the lowest blob number and hasn't been renumbered yet, that is below the 0 blob
for loop = 1:maxloops
    yblob(:,:,)=yblob(:,:,)+((maxloops+1+blobnumber)-yblob(:,:,).*(yblob(:,:,)==min(min(yblob(:,:,)))).* (yblob(:,:,)<maxloops+1);
    blobnumber=blobnumber+1;
end

yblob(:,:,)=yblob(:,:,)-maxloops-1;
numberofblobs=max(max(yblob));

% Calculate data
clear centroids blobarea majoraxis roundedness
for loop = 1:numberofblobs
    blobarea(loop)=sum(sum(yblob(:,:,)==loop));
    [yp,xp] = find(yblob==loop);
    xbar=mean(xp);
ybar = mean(yp);
centroids(loop,1:2) = [xbar ybar];
% Set centroid to 0,0 for each blob
yp = yp - ybar;
xp = xp - xbar;

sigxy = sum(xp.*yp);
sigxx = sum(xp.^2);
sigyy = sum(yp.^2);
axisone = (atan(-2*sigxy/(sigyy-sigxx))/2)/pi*180;

% Flip because y axis is flipped
axisone = 0 - axisone;

% Make axisone positive
axisone = axisone + 180*(axisone<0);

% Make axistwo perpendicular
axistwo = axisone - 90;

% Make axistwo positive
axistwo = axistwo + 180*(axistwo<0);

axis = cat(2, axisone, axistwo);

% Convert to radians for Matlab
radax = axis/180*pi;

% put in + sigxy below to compensate for inverse signed y axis
Iu = .5*(sigyy+sigxx) + .5*(sigyy-sigxx).*cos(2*radax) + sigxy.*sin(2*radax);

% major axis is the one that minimizes Iu
majoraxis(loop) = axisone*(Iu(1)<Iu(2))+axistwo*(Iu(1)>Iu(2));

% calculate roundedness
majorlength = 2*2^.5/blobarea(loop)^.5*(sigxx+sigyy+((sigxx-sigyy)^2+sigxy^2)^.5)^.5;
minorlength = 2*2^.5/blobarea(loop)^.5*(sigxx+sigyy-((sigxx-sigyy)^2+sigxy^2)^.5)^.5;
roundedness(loop) = minorlength/majorlength;

end
if numberofblobs == 0
    blobarea = 0; centroids = [0 0]; majoraxis = 0; roundedness = 0; numberofblobs = 1;
end
data = double(cat(2,rot90((1:numberofblobs),3),blobarea',centroids,
majoraxis',roundedness'));
celldata{fileloop} = data;
end

% Save data to file
fid=fopen(datafilename, 'w');
for loop=1:numberoffiles
  clear savedata
  savedata = celldata(loop);
  fprintf(fid,'%s
',filenames(loop,:));
  fprintf(fid, 'Blob Number,Area,X bar,Y Bar,Orientation,
Roundedness,
');
  fprintf(fid, '%4.0f,%8.0f,%4.3f,%4.3f,%3.3f,%3.3f
',savedata');
end
fclose(fid);
toc
disp('Processing finished.
')
disp(['Data in file: ',datafilename])
APPENDIX B

SAS PROGRAM CODE

SAS Program Code and Results for Nuclear Alignment Variance

// EXEC SAS
//SAS.SYSIN DD *
* option Nocenter;
title 'variance with flat ';
data cell1;
input substrate channel variance ;
TC=6*(a-1)+b;
*A is substrate, B is the channel length;
lines;
1 1 0.120025320
1 2 0.140548885
1 3 0.201267943
1 4 0.233743922
1 6 .
2 1 0.111432161
2 2 0.156181555
2 3 0.215173649
2 4 0.247597452
2 5 0.249211137
2 6 0.488828114
3 1 0.159604592
3 2 0.190951711
3 3 0.22957645
3 4 0.200737348
3 5 0.226694144
3 6 0.475349311
4 1 0.15159373
4 2 0.155321317
4 3 0.204336273
4 4 0.236086589
4 5 0.22625388
4 6 0.465452493
;
proc print;
proc glm;
class substrate channel;
model variance =substrate channel;
means substrate channel;
lsmeans substrate channel/pdiff=all cl adjust =Tukey alpha=0.05;
output out = cell2 PREDICTED = ypred RESIDUAL = z;
proc standard std =1.0;
var z;
proc rank normal=blom;
var z;
ranks nscore;
proc print;
;
proc plot;
plot z*ypred = '*' /vref=0 vpos=19 hpos =50;
plot z*nscore='*' /vref =0 href=0 vpos=19 hpos=50;
run;

Obs     substrate channel variance   TC   a   b
1        1         1       0.12003 .   .   .
2        1         2       0.14055 .   .   .
3        1         3       0.20127 .   .   .
4        1         4       0.18557 .   .   .
5        1         5       0.23374 .   .   .
6        1         6       .     .   .   .
7        2         1       0.11143 .   .   .
8        2         2       0.15618 .   .   .
9        2         3       0.21517 .   .   .
10       2         4       0.24760 .   .   .
11       2         5       0.24921 .   .   .
12       2         6       0.48883 .   .   .
13       3         1       0.15960 .   .   .
14       3         2       0.19095 .   .   .
15       3         3       0.22958 .   .   .
16       3         4       0.20074 .   .   .
17       3         5       0.22669 .   .   .
18       3         6       0.47535 .   .   .
19       4         1       0.15159 .   .   .
20       4         2       0.15532 .   .   .
21       4         3       0.20434 .   .   .
22       4         4       0.23609 .   .   .
23       4         5       0.22625 .   .   .
24       4         6       0.46545 .   .   .
The GLM Procedure

Class Level Information

<table>
<thead>
<tr>
<th>Class</th>
<th>Levels</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>substrate</td>
<td>4</td>
<td>1 2 3 4</td>
</tr>
<tr>
<td>channel</td>
<td>6</td>
<td>1 2 3 4 5 6</td>
</tr>
</tbody>
</table>

Number of observations 24

NOTE: Due to missing values, only 23 observations can be used in this analysis.

The GLM Procedure

Dependent Variable: variance

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>8</td>
<td>0.24086021</td>
<td>0.03010753</td>
<td>86.94</td>
<td>&lt;.0001</td>
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<tr>
<td>Error</td>
<td>14</td>
<td>0.00484832</td>
<td>0.00034631</td>
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<td></td>
</tr>
<tr>
<td>Corrected Total</td>
<td>22</td>
<td>0.24570853</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R-Square 0.980268  Coeff Var 8.119365  Root MSE 0.018609  variance Mean 0.229197

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type I SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>substrate</td>
<td>3</td>
<td>0.01808971</td>
<td>0.00602990</td>
<td>17.41</td>
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</tr>
<tr>
<td>channel</td>
<td>5</td>
<td>0.22277050</td>
<td>0.04455410</td>
<td>128.65</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Type III SS</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>substrate</td>
<td>3</td>
<td>0.00184427</td>
<td>0.00061476</td>
<td>1.78</td>
<td>0.1980</td>
</tr>
<tr>
<td>channel</td>
<td>5</td>
<td>0.22277050</td>
<td>0.04455410</td>
<td>128.65</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>
The GLM Procedure

Level of variance
substrate N Mean Std Dev
1 5 0.17623170 0.04597320
2 6 0.24473734 0.13122112
3 6 0.24715226 0.11469854
4 6 0.23984071 0.11600948

Level of variance
channel N Mean Std Dev
1 4 0.13566395 0.02351353
2 4 0.16075087 0.02137420
3 4 0.21258858 0.01280010
4 4 0.21749846 0.02916432
5 4 0.23397577 0.01072100
6 3 0.47654331 0.01173346

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

variance LSMEAN Number
substrate LSMEAN
1 0.22275834 1
2 0.24473734 2
3 0.24715226 3
4 0.23984071 4

Least Squares Means for effect substrate
Pr > |t| for H0: LSMean(i)=LSMean(j)

Dependent Variable: variance

<table>
<thead>
<tr>
<th>i/j</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2631</td>
<td>0.1905</td>
<td>0.4669</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.2631</td>
<td>0.9958</td>
<td>0.9674</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.1905</td>
<td>0.9958</td>
<td>0.9028</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.4669</td>
<td>0.9674</td>
<td>0.9028</td>
<td></td>
</tr>
</tbody>
</table>

variance substrate LSMEAN 95% Confidence Limits

<table>
<thead>
<tr>
<th>substrate</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.222758</td>
<td>0.204420</td>
<td>0.241097</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.244737</td>
<td>0.228443</td>
<td>0.261032</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.247152</td>
<td>0.230858</td>
<td>0.263447</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.239841</td>
<td>0.223546</td>
<td>0.256135</td>
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<td></td>
</tr>
</tbody>
</table>
### Least Squares Means for Effect substrate

<table>
<thead>
<tr>
<th>i</th>
<th>j</th>
<th>Difference</th>
<th>Simultaneous 95% Confidence Limits for LSMean(i)-LSMean(j)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>-0.021979</td>
<td>-0.055224 -0.011266</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>-0.024394</td>
<td>-0.057639 0.008851</td>
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<tr>
<td>1</td>
<td>4</td>
<td>-0.017082</td>
<td>-0.050328 0.016163</td>
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<tr>
<td>2</td>
<td>3</td>
<td>-0.002415</td>
<td>-0.033643 0.028814</td>
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<tr>
<td>2</td>
<td>4</td>
<td>0.004897</td>
<td>-0.026332 0.036125</td>
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<tr>
<td>3</td>
<td>4</td>
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<td>-0.023917 0.038540</td>
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</table>

#### The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

<table>
<thead>
<tr>
<th>channel</th>
<th>variance</th>
<th>LSMEAN</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.135664</td>
<td>0.115707</td>
<td>0.155621</td>
</tr>
<tr>
<td>2</td>
<td>0.160751</td>
<td>0.140794</td>
<td>0.180707</td>
</tr>
<tr>
<td>3</td>
<td>0.212589</td>
<td>0.192632</td>
<td>0.232545</td>
</tr>
<tr>
<td>4</td>
<td>0.217498</td>
<td>0.197542</td>
<td>0.237455</td>
</tr>
<tr>
<td>5</td>
<td>0.233976</td>
<td>0.214019</td>
<td>0.253932</td>
</tr>
<tr>
<td>6</td>
<td>0.471255</td>
<td>0.447642</td>
<td>0.494868</td>
</tr>
</tbody>
</table>

### Least Squares Means for effect channel
Pr > |t| for H0: LSMean(i)=LSMean(j)

<table>
<thead>
<tr>
<th>i/j</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4374</td>
<td>0.0005</td>
<td>0.0003</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.4374</td>
<td>0.0150</td>
<td>0.0075</td>
<td>0.0008</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.0005</td>
<td>0.0150</td>
<td>0.9988</td>
<td>0.5966</td>
<td>&lt;.0001</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.0003</td>
<td>0.0075</td>
<td>0.9988</td>
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<td>&lt;.0001</td>
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<td>0.5966</td>
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<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td></td>
</tr>
</tbody>
</table>

#### Least Squares Means for effect channel
Dependent Variable: variance

<table>
<thead>
<tr>
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<th>variance</th>
<th>95% Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.135664</td>
<td>0.115707 0.155621</td>
</tr>
<tr>
<td>2</td>
<td>0.160751</td>
<td>0.140794 0.180707</td>
</tr>
<tr>
<td>3</td>
<td>0.212589</td>
<td>0.192632 0.232545</td>
</tr>
<tr>
<td>4</td>
<td>0.217498</td>
<td>0.197542 0.237455</td>
</tr>
<tr>
<td>5</td>
<td>0.233976</td>
<td>0.214019 0.253932</td>
</tr>
<tr>
<td>6</td>
<td>0.471255</td>
<td>0.447642 0.494868</td>
</tr>
<tr>
<td>i</td>
<td>j</td>
<td>Difference Between Means</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>--------------------------</td>
</tr>
<tr>
<td>1</td>
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<th>Obs</th>
<th>substrate</th>
<th>channel</th>
<th>variance</th>
<th>TC</th>
<th>a</th>
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Plot of $Z^{\text{ypred}}$. Symbol used is '***'.

NOTE: 1 obs had missing values. 1 obs hidden.

Plot of $Z^{\text{nscore}}$. Symbol used is '***'.

NOTE: 1 obs had missing values.
SAS Program Code and Results for Actin Alignment Variance

// EXEC SAS
//SAS.SYSIN DD *
option Nocenter;
title 'variance with flat ';
data cell1;
input substrate channel variance;
TC=6*(a-1)+b;
* A is substrate, B is the channel length;
lines;
1 1 0.262869898
1 2 0.309569191
1 3 0.371645292
1 4 0.365178837
1 5 0.391137846
1 6 0.456642384
2 1 0.267027588
2 2 0.308092557
2 3 0.37484491
2 4 .
2 5 0.345648581
2 6 0.450501434
3 1 0.256872869
3 2 0.256035571
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3 4 0.321308816
3 5 0.318110155
3 6 0.462657405
4 1 0.26118244
4 2 0.275303582
4 3 0.325014329
4 4 0.328224658
4 5 .
4 6 0.439531348
5 1 0.216228965
5 2 0.249224738
5 3 0.354704116
5 4 0.293544916
5 5 0.314562527
5 6 0.460954775;
proc print;
proc glm;
class substrate channel;
model variance =substrate channel;
means substrate channel;
lsmmeans substrate channel/pdiff=all cl adjust =Tukey alpha=0.05;
output out = cell2 PREDICTED = ypred RESIDUAL = Z;
proc standard std =1.0;
var z;
proc rank normal=blom;
var z;
ranks nscore;
proc print;
proc plot;
plot z*ypred = '*' / vref=0 vpos=19 hpos =50;
plot z*nscore= '*' / vref =0 href=0 vpos=19 hpos=50;
run;

variance with flat

07:57 Friday, October 10, 2003   1

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The GLM Procedure

Class Level Information

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Number of observations 30

NOTE: Due to missing values, only 27 observations can be used in this analysis.

The GLM Procedure

Dependent Variable: variance

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R-Square 0.940864  Coeff Var 6.245709  Root MSE 0.020499  variance Mean 0.328202

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The GLM Procedure

Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

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Least Squares Means for effect Substrate

Pr > |t| for H0: LSMEAN(i)=LSMEAN(j)

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Least Squares Means for Effect substrate

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The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey-Kramer

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Least Squares Means for effect channel
Pr > |t| for H0: LStat(i) = LStat(j)

Dependent Variable: variance

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Least Squares Means for Effect channel

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Plot of $Z*\text{ypred}$. Symbol used is '**'.

NOTE: 3 obs had missing values.

Plot of $Z*\text{nscore}$. Symbol used is '**'.

NOTE: 3 obs had missing values.
WORKS CITED


Li, M. PhD. Dissertation, Louisiana Tech University, 2003.


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