MICROPATTERN TRACTION MICROSCOPY:
A TECHNIQUE FOR THE SIMPLIFICATION OF CELLULAR TRACTION
FORCE MEASUREMENTS

by

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DEDICATION

I dedicate this work to my family, in particular my parents for their encouragement and guidance throughout these years.
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ABSTRACT 

Cells respond to a number of cues that affect how they interact with their surrounding environment, such as topology, the presentation of adhesive ligands, and stiffness. Recent advancements in the field of mechanobiology have revealed that one of the main ways in which cells sense these cues is through contractile forces. Mechanobiology research seeks to understand how environmental cues affect the forces that cells exert on their surrounding environment and how these mechanical forces are communicated to the cell and transformed into biochemical signals. Therefore, quantitative methods have been developed to determine cell contractility on soft, optically transparent, deformable surfaces by quantifying substrate deformation in terms of cellular traction forces. However, the currently available tools that are used to study cell interactions are limited in their applicability due to the need for specialized technical expertise that is not amenable to the widespread adaptation of these techniques. Therefore, we have sought to develop a novel traction force microscopy technique known as micropattern traction microscopy. With this technique, we hope to greatly simplify the
current traction force microscopy techniques and provide a method which will be able to be adopted by a wide range of laboratories.

This dissertation describes the process of the development and application of this novel traction force technique to probe questions in mechanobiology that have not been previously broached due to the lack of appropriate tools. The technique itself uses indirect microcontact printing to create a regularized array of fluorescent protein onto a glass substrate, which is then transferred to an optically transparent, soft, elastic polyacrylamide hydrogel. Cells, limited by their ability to adhere only to patterned regions, will deform the pattern at these defined points. Thus, with knowledge of the bulk elastic properties of the substrate and a priori knowledge of the pattern, we are able to quantify the force a cell is exerting without its removal. We also developed and released a robust, automated MATLAB program that will aid users in the calculation of traction forces so that people with limited experience with programming can utilize the program without significant investments into training. This indirect approach allows for not only individual proteins, but also for multiple, spatially distinct, fluorescent proteins such as fibronectin and gelatin to be simultaneously patterned onto this surface as well. The ability to pattern multiple proteins in a spatially defined region significantly aids in giving users control over as many parameters as possible. Finally, we will explore the current and future potential that this technique has to offer to researchers in the field of mechanobiology.
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LIST OF ABBREVIATIONS

2-D ................................................................. 2-Dimensional
3-APTMS ..................................................... (3-Aminopropyl)trimethoxysilane
3-D ............................................................... 3-Dimensional
AFM .............................................................. Atomic Force Microscopy
BEM ............................................................. Boundary Element Method
BU ................................................................. Boston University
CDF .............................................................. Cumulative Distribution Function
CNRS .......................................................... Centre National de la Recherche Scientifique
CTF .............................................................. Cell Traction Force
DI ................................................................. Deionized Water
ECM .............................................................. Extracellular Matrix
IF ................................................................. Intermediate Filaments
F-Actin .......................................................... Filamentous Actin
FA ................................................................. Focal Adhesion
Fn ................................................................. Fibronectin
FTTC ............................................................. Fourier Transform Traction Cytometry
FRET ............................................................. Forester Resonant Energy Transfer
MEF ............................................................. Mouse Embryonic Fibroblast
ICAMs .......................................................... Intercellular Adhesion Molecules
mPADS ........................................................ Microfabricated Post-Array-Detectors
MTM ............................................................. Micropattern Traction Method
NA ................................................................. Numerical Aperture
NIH .............................................................. National Institute of Health
NHS-ester ......................................................... Acrylic Acid N-hydroxysuccinimide Ester
PAA ................................................................. Polyacrylamide
PEG ................................................................. Polyethylene Glycol
PDMS .............................................................. (Polydimethyl)siloxane
RSC ................................................................. The Royal Society of Chemistry
TEMED ......................................................... Tetramethylethylenediamine
TFM .............................................................. Traction Force Microscopy
TRFP .............................................................. Traction Reconstruction with Point Forces
UV ................................................................. Ultraviolet
CHAPTER 1: INTRODUCTION

1.1 Why Study the Mechanical Cell?

Cells are living machines that integrate and respond to many different types of signals in their environment. They are constantly receiving soluble, mechanical, and electrical cues that inform how they will respond to their current environmental conditions. These signals are a critical part to all biological systems as they aid in organizing and instructing cellular behavior. Though chemical signaling is crucial to cell viability, mechanical signals and composition of the extracellular environment have been found to play significant roles in the behavior of cells as well. In fact, it had been recognized over 100 years ago that this was a potential mechanism through which embryos developed; however, interest in biological signaling and pathways overshadowed this area of research (Thompson, 1917).

There has been a recent increase in interest in mechanotransduction, which is the field of study that seeks to understand the mechanisms by which cells are interpreting physical, mechanical signals and converting them to biochemical ones. That interest has also spawned a greater need for quantitative ways in which to measure how cells interact with the complex compositions of in vivo environments as all cells in the body mechanically interact with the surrounding environment, known as the extracellular matrix (ECM), as well as with other cells. Cells are directly, physically interacting via transmembrane molecular structures, which help to transmit physical signals of the surrounding environment into biochemical signals as well as aid in force transmission to the cytoskeleton (CSK). Figure 1.1 shows the various ways in which a cell can interact
mechanically with its environment (Eyckmans et al., 2011). The mechanical factors of the environment that cells have been found to be able to respond to that can be sensed through these pathways include substrate stiffness, cell shape, fluid shear stress, and mechanical stimulation (Discher et al., 2005; Eyckmans et al., 2011; Watt et al., 1988).

CTFs (cellular traction forces) are critical to understanding the functioning of cells, so gaining a quantitative understanding of this aspect of cell behavior and how cells interrogate the extracellular environment is of great importance. Contractility through focal adhesions (FAs) is the main way in which cells probe and sense the surrounding mechanical environment of the cell. Cues such as the stiffness of the substrate and cell spread area are responded to by the cells through the ordering of the cytoskeleton, which closely links these aspects to cellular contractility. Through inhibiting contractility, cells no longer receive feedback or to respond to these cues in different manners, causing cells to not respond to changes in substrate rigidity and alter their shape (Engler et al., 2004; McBeath et al., 2004).

Cell contractility is involved with a number of critical cell processes that range from basic functions such as cell division and the migration of cells in inflammatory processes to wound healing and determining the fate of stem cells. In fact, there is a growing appreciation of how many diseases, such as asthma and metastatic forms of cancer, may be related to how and what cells are interpreting as their surrounding environment (Kraning-Rush et al., 2012a; Kraning-Rush et al., 2012b; Lavoie et al., 2009). With this in mind, we believe that determining CTFs is an important measure of how cells are interpreting their surrounding environment and an indicator of cell health.
1.2 Cell Traction Force Generation and the Cytoskeleton

One feature common of many cell types is that they have structural proteins that help to organize their internal structures as well as interact with their surrounding environments. These structures together loosely comprise what is known as the CSK, the force exerting component of which is composed largely of actin and nonmuscle myosin II fibrils (Figure 1.2.1) (Geiger et al., 2001; Polio and Smith, 2014). There are a number of different types of adhesion proteins and junctions which cells use to physically adhere to their environment. The relative ability of the proteins to exert force depends on the protein’s adhesion strength as well as the structure of the proteins within the cell that connect them to the CSK.

The CSK of the cell is composed of several main proteins filamentous actin (F-actin), microtubules, and intermediate filaments (IFs) (Fletcher and Mullins, 2010). F-actin in combination with myosin comprises the majority of the force producing cytoskeletal elements. The F-actin-myosin contractile assembly uses ATP to pull F-actin fibers past one another, resulting in the production of force at the ends of the F-actin through a sliding mechanism. This sliding movement results in the generation of contractile forces. The force exerted by a single myosin II head on an actin filament is in the range of 3-4pN; however, the ensemble produces forces on the order of nN due to the bundling of multiple F-actin fibers at the sites of force application (Finer et al., 1994). The biochemical feedback mechanism by which cells respond, often times at or near the site of the adhesion point, defines the concept of mechanotransduction.

Cells form direct adhesions to their substrates or alternatively to other cells via a
number of different types of receptor proteins. Different classes of proteins are responsible for cells forming each type of adhesions and subsets of those types of adhesions can vary depending on the exact type of protein being adhered to. Cell-ECM adhesions are generally formed by integrins, which are composed of $\alpha$ and $\beta$ subunits. These subunits combine to form heterophilic adhesions to various different types of ECM proteins. Similarly, there are cell-cell adhesions that can be formed by cadherins. These are homophilic adhesions as both cells must possess the same type of cadherins in order to bind to one another. The importance of these types of adhesion classes is that they are directly linked to the CSK via adhesion complexes to F-actin and have been implicated in helping to actively transduce contractile force from the CSK to the ECM and other cells. In response to these contractile forces, traction forces arise at the cell-ECM and cell-cell junctions.

Passively, there are a number of other different classes of adhesions that do not take active roles in exerting CTFs, but still play an important role in cell adhesions. Desmosomes, which are attached to IFs, help to distribute force passively throughout tissues, but have not been found to place an active mechanical role. There are also a number of cell-cell adhesion molecules known as intercellular adhesion molecules (ICAMs) that have not been shown to interact actively with the CSK, yet seem to be regulated via the activation of the integrin subunits (Murikipudi et al., 2012). These types of interactions where there is co-regulation or crosstalk between integrins, cadherins, and other types of surface proteins are increasingly of interest to the mechanotransduction community.
1.3 Cellular Traction Force Measurement Techniques

CTFs are the active forces exerted by cells on their surrounding environment. These forces are exerted by the cell’s CSK network onto the substrate via membrane proteins such as integrins and cadherins, as well as other transmembrane proteins (Section 1.2). These forces are of importance due to their involvement in a number of biological processes. Techniques to quantitatively measure how much force a cell is placing onto its environment have been of great interest to the scientific community.

A fundamental way in which measurements of force are able to be made are through the deformation of a known material. One of the earliest methods used to attempt to quantitatively measure CTFs was the observation of wrinkles in a thin film of elastomer, (Polydimethyl)siloxane (PDMS) (Figure 1.3.1) (Harris et al., 1980). It was found that when placed on such surfaces, cells would result deform the softer substrates due to contractions that the cell exerted. As PDMS is optically transparent, these deformations could be observed using traditional light transmission microscopy. In order to convert these deformations into forces, micropipettes were used to deform the substrate. The deflection of the micropipettes gave an estimate of the amount of force that a cell would exert on the surface given a similar deformation. The problem with this technique was that this technique was highly qualitative in attempting to convert the deformations into forces and presented a mathematically intractable problem.

Polyacrylamide (PAA) substrates provided the answer to the issue with elastomeric substrates in that were optically transparent, soft, highly elastic, and could be modified with protein through surface chemistry modifications to allow better adhesion
of cells (Pelham and Wang, 1997). Therefore, micron-sized fluorescent beads were introduced in order to determine the deformation of the substrate (Lee et al., 1994; Oliver et al., 1995). After the cells were removed from the substrate or moved past a particular area, the deformation from the original position could be determined. The problem with this technique however was that the surface was not very amenable to cell adhesion still and that the deformations were not completely elastic (Figure 1.3.2). A more elastic substrate that could be modified with protein was necessary. Although the initial techniques used a low density of beads and physical deformation of the gel to determine cell traction forces, advances in computational models of the cell traction forces allowed for a higher density of beads to be observed and tracked within the deformed PAA gel (Figure 1.3.3) (Dembo and Wang, 1999; Munevar et al., 2001). This technique, known as the boundary element method (BEM), uses a mathematical field approximation as well as knowledge of the material properties of PAA to determine the CTFs through the deformation of the beads.

Generally, this technique requires the inversion of a large matrix and then solving a large number of linear equations, which can be sensitive to noise in the system (Sabass et al., 2008). Techniques such as Fourier-transform traction cytometry (FTTC) have been developed to simplify the calculations and speed up computational time (Butler et al., 2002), but these techniques are not real-time and require the careful removal of the cells post-experiment, which sets practical limitations on the use of the technique in terms of time and number of samples that can be acquired in a given timeframe (Sabass et al., 2008; Stricker et al., 2010).
Another popular technique for measuring CTFs was the development of microfabricated post-array-detectors (mPADs) (Tan et al., 2003). The mPADs are created using photolithography and replica molding is used to create the micropillars out of an elastomer, PDMS. As can be seen in Figure 1.3.4, the cells can deflect the elastomeric mPADs by deflecting them. Using a simple beam equation, the deflection of an individual beam can be correlated to the amount of force that the cell is placing on it (Fu et al., 2010). This technique vastly simplified the calculations required to determine CTFs and provided some unique opportunities as compared to contemporary techniques in that registration of multiple images was not required and samples could be observed at many different time points with cell-ECM proteins ranging from collagen and fibronectin (Fn) to cell-cell proteins such as cadherins (Ganz et al., 2006). The wide variety of tools that this presented to the community represented a great advancement in 2D CTF calculations. Variations on the technique allowed for cell-cell tractions could be measured through the use of micropatterning onto the mPADs themselves as well.

Other variations on these techniques have been presented as well to determine cell-cell and cell-ECM traction forces. For example, calibrated deformations of fluorescent micropatterned shapes can be used as an alternative technique to find CTFs of cells (Tseng et al., 2011). Determining the deformation of the micropatterned shapes revealed that CTFs could be measured without the need for beads being present for each measurement, thus simplifying the traction force calculations as a priori knowledge of the patterns would give the initial pattern. This technique is more interested in finding the overall traction force of the cell itself rather than at individual FAs, but provides a unique...
method for determining these forces for individual cells.

CTFs can be determined in 3D as well, but this represents much more challenging computational and technical hurdles due to the 3D imaging and tracking of beads as well as attempting to exert control over many variables. As properties such as matrix stiffness are altered, features such as the pore size of the material as well as the ligand density that the cell comes into contact with can change significantly. In order to alter each property independently, PEG hydrogels have been engineered to perform such tasks. Cells embedded within the hydrogel deform the material around them and the deflection of fluorescent beads can be measured in much the same way as with 2D hydrogels after removal of the cell (Koch et al., 2012; Legant et al., 2013). Semi-quantitative 3D techniques have been developed to try to look at these deformations through the contraction of collagen gels (Gjorevski and Nelson, 2012; Kraning-Rush et al., 2011; Legant et al., 2012). These techniques look at the overall contraction of the group of cells and determine the amount of force exerted on the gel through measuring the change in size of the gel, fiducial markers within them, or even the deflection the cell exerts on a large PDMS posts. However, they only provide an approximation of the forces exerted by the sample as a whole.

The fundamental unifying factor of all traction force microscopy (TFM) techniques is that they observe deformations of a material and thorough knowledge of that material’s physical properties. CTFs of individual or groups of cells can then be determined through image analysis techniques. The different approaches they take have different strengths and weaknesses depending on if the goal of the technique is to look at
individual FAs (TRFP or mPADs) versus the force exerted by the whole cell as a field (BEM, FTTC, and micropatterned shape deformation). Improving upon the versatility of these techniques to look at more features and would give a valuable tool to the communities that are interested in determining CTFs on different substrates.
1.4 Soft Lithography and Microcontact Printing

The ability to obtain sub-cellular, regularized arrays would greatly simplify the process of determining the forces cells exert on substrates. In order to achieve such features, soft lithography and micropatterning can provide tools to create such features. Soft lithography is a technique derived from the microelectronics industry to create replica molds of a hard material using a soft elastomer (Xia and Whitesides, 1997). Traditionally, PDMS is used to replicate microscale features that were created on a hard silicon mask. The benefit of this technique is that micron-scale features can be made and replicated multiple times using this replica molding technique, thus saving time and expense in making devices.

The steps to the photolithographic technique are shown in Figure 1.4.1 (Whitesides et al., 2001). The basic technique is very similar to developing a photograph. A photomask with the desired negative or positive features (depending on the process) is created using either e-beam writing onto a chrome mask or the creation of a film photomask. Depending on the type of photoresist that is on the silicon substrate, this may be either the positive or the negative of the image on the photomask. Exposure of the mask and substrate, which are placed in close proximity to one another, to UV light causes the polymerization of the negative photoresist on the substrate. The unpolymerized photoresist can then be removed to reveal the features. Feature size is limited by diffraction of the light due to the numerical aperture (NA) of the lens, the wavelength of light used, and the optical properties of the photoresist itself to obtain features can be obtained down to $\sim 100\text{nm}$. 
In order to create a replica of the pattern, PDMS is poured into the mold and cured. The cured elastomer can be removed to reveal a relief pattern, a “stamp”. The replica mold itself can then be utilized in a variety of functions ranging from the simple patterning of proteins onto glass to microfluidic devices. The patterned proteins can be used to control the shape and alignment of individual or multicellular structures when patterned on a non-fouling substrate such as PAA or gold that can be functionalized to prevent cell adhesion to areas lacking the protein pattern (Figure 1.4.2) can limit the adhesion of cells to the desired pattern (Mrksich et al., 1997).

Having control over the arrangement of single and multi-protein patterns can allow for the interrogation of cells in response to the presence of one or multiple proteins in addition to control over cell shape and alignment. It is critically important to be able to exert control over these properties when studying features that regulate the CSK. Factors controlling cell shape, such as the patterned area over which the protein is able to spread, have been found to be one of the co-determinants in stem cell fate as well as have control over some functional properties of cells (Guilak et al., 2009; McBeath et al., 2004; Singhvi et al., 1994).
1.5 Improving Upon Current Cell Traction Force Techniques

Current CTF measurements techniques are based on the principle that cells can apply forces that will physically deform a material and those physical deformations will be optically detectable. Each TFM technique has its own unique applications as well as drawbacks in trying to determine CTFs. Many of these are technical in nature while some are a result of the technique itself. In order to improve upon the previous techniques, we need to have an understanding of what these shortcomings are. An ideal technique would be technically simple in order that the technique is feasible for many different laboratory settings, robust and repeatable, and allow for the use of multiple different types and classes of proteins.

In determining CTFs, studying how cells apply force is one of the most critical aspects to determining how forces should be measured. CTFs are applied by cells at punctate points at the FAs at cell-ECM junctions (Ch 1.1). If studies are interested in looking at the traction force of a whole cell, it may not be critical to study how the force of an individual FA is changing. Therefore, a technique such as BEM or FTTC may be appropriate to calculating the CTF of a cell overall. If one was to look at traction forces exerted at individual FAs, a techniques involving mPADs or TPFR would be ideal; however, they require additional microfabrication of elastomeric stamps. In the case of mPADs, the data is also not directly comparable to other techniques.

By changing the surface area of contact, the CTFs change significantly as cells are limited to which areas they can adhere (Han et al., 2012). TPFR and mPADs represent ideal techniques for the determination of tractions of individual FAs, but due to the fact
that the area in which cells are in contact with can change based on the diameter of the micropillars and the micropatterned adhesion points, they are not directly comparable to techniques that require the even coating or patterning of continuous areas of protein.

These techniques also have fundamental technical drawbacks in their execution. Techniques that use PAA hydrogels require the removal of cells at the conclusion of the experiment to determine the initial pattern of the fluorescent beads which had been randomly dispersed throughout the gel initially. Without this initial image, there cannot be any calculation of the displacement of the beads in the substrate. This fundamentally limits the experiment to being utilized for shorter time periods. Cells cannot be moved from the experimental platform until the conclusion of the experiments without causing a disruption in the registration of the displaced and undeformed images. Therefore, this technique severely limits the throughput of data that can be acquired in a given time period per a given piece of equipment. Also, due to this requirement that the cells be removed, the CTFs remain unknown until the conclusion of the experiment, when an off-line data analysis can be conducted. A technique that allows for the analysis of data prior to this point would be very beneficial to determine if the experimental conditions are appropriate and that the experiments had been prepared correctly. This type of analysis can be provided by techniques such as mPADs and micropatterned shapes, but this comes at a cost.

Technical limitations on the adaptability of beads within PAA to different techniques spawned techniques like mPADs which allowed for much faster analysis without some of the severe drawbacks such as cell removal and complex traction force
calculations. mPADs and calibrated micropatterns allow for the real time analysis of CTFs without the need to remove cells as the patterns are known \textit{a priori}. These patterns can therefore be quickly compared to the known patterns to calculate CTFs. This allows for experimentalists to perform studies on multiple samples and regions of the sample at the same time without worrying about the registration of the initial and final images. Samples can be removed from the visualization equipment and individual samples can be measured over a much longer period of time.

The degree of material fouling can be an issue for CTF techniques. With longer time, materials such as PDMS tend to foul and cells may attach to areas that were unintended. This can be a problem since cell attachment to the side of mPAD posts would affect the CTF calculations being made. This is one area in which PAA hydrogels excel as PAA is very non-fouling and will resist the adhesion of ambient protein for extended periods of time.
1.6 Aims and Objectives

The goal of this project is to create a novel, improved TFM technique that will allow us to determine CTFs simply and to give the users a high degree of control over the desired experimental parameters. The research done in this project will help to develop a deeper understanding of how cells respond to changes in substrate stiffness, ligand presentation, and substrate dimensionality by using a single mechanical system to determine the effect of environmental cues as they relate to CTFs.

The project will seek to develop and implement this novel technique upon which future research for studying the mechanical environment of cells will be based upon. Previous techniques for measuring CTFs have used methods demanded highly specialized microfabrication techniques or required the use of complex mathematics which inaccurately represent CTFs. The CTF system that was developed consists of a regularized array of fluorescently labeled protein markers on an optically transparent PAA hydrogel. Regularization of patterning will greatly simplify the CTF calculations by limiting adhesions to the fluorescently labeled protein areas, allowing for real time, rapid analysis of the experimental results. The system will be able to be used for multiple proteins and adhesion molecules with the ability to easily tune the stiffness of the hydrogel and spread area of both individual and groups of cells.

There are three major aims of this project therefore to demonstrate the technique and its robustness in determining CTFs:

1) **Develop a method for measuring traction forces on a regularized pattern of fluorescently labeled protein dots on a polyacrylamide surface.**
The goal of this method is to develop a novel CTF technique that will address a number of the shortcomings of previous techniques by patterning a regularized array of fluorescently labeled protein dots 1-2μm in diameter. This will serve as the foundation for the project and allow previously unexplored questions to be addressed. As part of this foundation, we will demonstrate that the experimental technique is mechanically sound and that the assumptions made in calculation of the forces are valid. The displacements of the pattern will be analyzed by a MATLAB program and converted into tractions exerted by cells.

2) Determine how different patterned ligands affect the response of cells to substrate.

The composition of ECM between tissues in the body is known to vary and therefore it is important to determine how cells interact with different ligand mixtures and cellular environments. It has been found that different or multiple cell-cell and cell-ECM interactions will cause different amounts of traction to be applied to the substrate. Micropatterning different ligands onto the same substrate will also help to prove the versatile nature of the developed technique and demonstrate how cells interact in an environment with different ligands. To achieve this, we patterned fluorescently labeled gelatin and Fn and analyzed the CTFs exerted by cells on both patterns.
Figure 1.1.1. The Mechanotransductive Cell.

This figure illustrates the various ways in which a cell can interact with its surrounding environment. Cells can interact with their surrounding via (A) cell-cell contacts connecting to the cytoskeleton, (B) through fluid flow or membrane deformation, (C) mechanical coupling to the nucleus of the cell, and (D) at cell-ECM contacts through the focal adhesions. Adapted from (Eyckmans et al., 2011). Reprinted under open source license from Elsevier.
Figure 1.2.1. Focal Adhesion of the Cell.

Focal adhesions are one type of junction at which the cell CSK interacts with the surrounding ECM via integrins. Filamentous actin (F-actin) undergoes stress due to the pulling by myosin II. Reprinted with permission from Macmillan Publishers Ltd (Geiger et al., 2009).
Cells can exert force on their substrates through contractile mechanisms. Techniques to observe how thin PDMS films were deformed were amongst the first techniques to be utilized to attempt to measure cell contractile forces. Reprinted from (Harris et al., 1980) with permission.
Figure 1.3.2 Cell Traction and Deformation on Silicone Substrates.

(A) Cells placed onto a silicone substratum with embedded latex beads were adopted to (B) better quantitatively measure the amount of force applied by the cells based on their deformations. (C) This deformations of the silicone were not completely elastic and did not recover to their original positions in many cases. Reprinted from (Lee et al., 1994) under the Creative Commons license.
Figure 1.3.3 Boundary Element Method of Cell Traction Forces

(A) The BEM method utilizes randomly dispersed fluorescent beads within a PAA hydrogel to (B) produce a displacement field after the removal of the cell. (C) The field of traction stresses is expressed as discretized vectors and (D) is colorized according to the stress. Adapted from (Munevar et al., 2001). Reprinted with permission.
Micropillar-array detectors (mPADs) are used to determine the amount of force which a cell exerts on the substrate via the deflection of microposts. (A) Post height and geometry can be varied to result in different stiffnesses of the substrate. A taller post makes the substrate more flexible and result in greater deflection for a given force. (B) A SEM image of a cell on mPADs can be seen deflecting the posts. (C) These deflections can then be turned into force measurements. Adapted from (Fu et al., 2010). Reprinted with permission.
Figure 1.4.1 Soft Lithography.

This figure demonstrates the general process of soft lithography. (A) A rigid photoresist master is created using photolithography. (B) An elastomer such as PDMS is then cast and cured on the master. (C) After removal, the stamp retains the features of the master. Adapted from (Whitesides et al., 2001). Reprinted with permission.
Figure 1.4.2 Microcontact Printing onto Gold.

Cell spread area and shape were controlled through the use of (A) microcontact printing onto a gold surface to create hydrophobic regions to where fluorescent fibronectin adhered. (B) As a result, cells only adhered to the region that was patterned to allow the adhesion of Fn. Adapted from (Kane et al., 1999). Reprinted with permission from Elsevier.
CHAPTER 2: METHODS

2.1 Fabrication of Silicon Masters and PDMS Stamps

The first step of the process of micropatterning is the creation of silicon masters from which to create the PDMS stamps. There have been a number of articles that have discussed this technique; however, it will be highly detailed to provide sufficient documentation of the design and technique used for this procedure (Sniadecki and Chen, 2007).

Circular dots of 1 – 2 μm in diameter were first designed using AutoCAD 2013 (Autodesk). The designs were then transferred to a chrome mask (Nanofilm). The mask is 5”x5”x0.09” in size and is made of a soda lime glass with a low reflective coating. The process for creating the masks following the BU Photonics Center Protocol (http://www.bu.edu/photonics/):

1. After calibrating the 2mm write head of the DWL66 mask writer, the mask is etched using the recommended power settings.

2. The photomask is developed using AZ1518 for 90 sec and then rinsed with DI water.

3. The photomask is then dried.

4. The chrome is then etched using a chrome mask etchant for 180 sec.

5. The mask is then rinsed with DI water and then dried.

6. The resist is then stripped from the photomask using 1165 Stripper at 75°C for 10 min.

7. The mask is rinsed with DI water and then dried.
Using this chrome mask, we then could create the silicon wafer using photolithography. The features of the silicon wafer were designed in such a manner as to not exceed the 5:1 ratio of feature height:width. The size of the stamps is 1.5 x 1.5 cm².

The procedure was carried out in a class 1000 cleanroom using the following steps:

1. Silicon wafers were cleaned using Piranha solution (1:3 30% hydrogen peroxide : 98% sulfuric acid) for 2 hrs.
2. Wafers were thoroughly rinsed with DI water in an automated water bath and then dried using a spin drier under nitrogen. They could then be stored for later use.
3. Wafers were cleaned with a sequence of ethanol, methanol, and isopropyl alcohol, and then dried under nitrogen.
4. Dried wafers were baked for 15 min at 200°C to remove excess water from the wafer.
5. Wafers were allowed to cool for 15 min before starting the procedure.
6. SU-8 5 was poured onto the surface of the silicon wafer at rest.
7. The wafer was spun from 0-500 rpm at 100rpm/sec and then ramped to 2700rpm at 300rpm/sec and held for 30 sec.
8. The wafer was baked for 1.5 min at 60°C and then 4 min at 90°C.
9. After baking the wafer was exposed to UV light using an MA6BA6 Karl Suss mask aligner to impart 120 J of energy using the lamp.
10. The wafer was then baked for 1 min at 60°C and 1 min at 90°C.
11. After baking, the wafer was placed into photoresist developer for 1 min to remove the excess resist.
12. The wafer is then hard baked at 150°C for 10 min to secure the pattern.

After hard baking, the wafer can then be used to create stamps. Sylguard 185 elastomer (PDMS) is poured into the molds and degassed for 30 min. The molds are then baked at 80°C for 2 hrs to cure the PDMS. After cooling, the PDMS stamp can be removed and used to stamp the surfaces.
2.2 Micropatterning a Single Pattern

Microcontact printing is the process by which the protein will be transferred from the PDMS stamp's pattern in the previous section to the coverslip. This process incorporates similar techniques to those that have used micropatterning previously and reviewed in a number of articles (Eichinger et al., 2012; Ruiz and Chen, 2007; Shen et al., 2008a; Shen et al., 2008b; Xia and Whitesides, 1997). The current process is illustrated in Figure 2.2.1 for a single micropatterned substrate. The micropatterns used were 1-2μm in diameter and 5-8.5μm in distance from center-to-center. The circular features were designed with previous techniques for microcontact printing and with similar techniques of cells adhering to distinct points, such as mPADs, in mind (Balaban et al., 2001; Maloney et al., 2008; Sniadecki and Chen, 2007). The patterning process here sought to transfer fluorescent Fn or porcine gelatin protein from the raised portion of the pattern to the glass in order to create dots that could then be transferred to a PAA hydrogel. The process for micropatterning using the PDMS stamps as created previously is described here in detail:

1. After removal from the mold, the PDMS stamp is plasma cleaned for 30 sec in a Harrick Plasma cleaner on high using air as the gas.
2. 150μl of fluorescently labeled protein solution is added to the surface. Depending on the protein and experiment, this concentration can range from 50 to 100 μg/ml.
3. The protein solution is incubated on the plasma treated stamp for 30 min under tin foil to avoid photobleaching.
4. After incubation, the excess solution is removed from the stamps and the stamp is dried.

5. The stamp is then placed in contact with glass coverslips that have been sonicated in ethanol and water and plasma treated for 1 min.

6. After 15 min of contact, the stamp is then removed and the patterns are visualized using a fluorescent microscope to determine if they have been transferred to the glass coverslip with sufficient fidelity.

7. After microcontact printing, the pattern can then be transferred to a PAA hydrogel immediately or stored under sterile PBS for future use.
2.3 Micropatterning Multiple Patterns

Multiple micropatterns can be performed by aligning the patterns of multiple stamps. Complex devices and techniques have been developed to align stamps; however, it is possible to make simplifications in order that good alignment can be achieved without the use of such devices and on the scale of 1-2μm over large areas (Mei et al., 2008). The use of differently spaced patterns to create a Moiré pattern (Figure 2.3.1) aids in this as instead of aligning patterns to obtain precisely spaced dots, all that needs to be aligned are the axes of the patterns. To create these aligned patterns of multiple proteins, the same procedure to pattern is followed as in section 2.2 for the initial pattern.

Figure 2.3.2 shows the procedure for adding an additional pattern to the substrate. Before the removal of the first pattern, the PDMS pattern can be aligned with a template underneath the glass coverslip. A piece of tape or line drawn on a surface can serve as an alignment marker for one border of the stamp, which can be observed through the stamp. Then, careful removal and alignment of the second pattern on top of the alignment marker can provide sufficient alignment for the patterns. Through the use of a Moiré pattern, this allows for the interference patterns to periodically align given the slightly different spacing of the patterns. In our case, we used 8.5μm and 8.45μm center-to-center differences to align the patterns.
2.4 Creation of Micropatterned Islands

Using a procedure similar to that described in section 2.1, molds for creating square islands of varying sizes were created using Su-8 25. An emulsion film mask was designed in CAD and developed by CAD / Art Services, Inc. as the smallest designed features were 10 μm in size. These masks were designed in order that the raised sections would come into contact with areas that were patterned, but desired to be removed. The removal of the pattern would prevent cells from adhering to the areas in which there was a lack of protein, just as if there had been no pattern there initially. Squares with sizes ranging from 15 x 15 μm² to 100 x 100 μm² were created using this technique.

The removal of the patterned protein was performed by placing a glutaraldehyde treated PDMS stamp in contact with the glass coverslip. Glutaraldehyde aids in the removal of the protein in the patterned areas by forming covalent bonds to the protein in the affected areas. Glutaraldehyde treatment of the PDMS stamp occurs in the following manner. The PDMS stamp is removed from the mold after curing for 2 hrs. They are then immediately plasma cleaned using a Harrick plasma cleaner on high for 30 sec. After plasma treatment, the PDMS is functionalized through the use of (3-aminopropyl)trimethoxy silane (3-APTMS). The stamp’s surface is treated with a thin layer of 5% solution of 3-APTMS for 5 min and then allowed to dry. After drying, the stamp is rinsed with water and submerged in a 2.5% solution of glutaric dialdehyde for 30 min. These stamps can be prepared ahead of the experiment. After stamping the desired pattern with the desired fluorescent protein, the protein is removed by placing the
stamp in contact with the glass coverslip. As can be seen in Figure 2.4.1, the stamp will only remove protein in which it comes into contact with.
2.5 Functionalizing Coverslips

The device that is utilized for this procedure requires that the PAA hydrogel adhere to the bottom coverslip. This is to prevent the possibility that the hydrogel could detach after the top coverslip is removed from the polymerized hydrogel and the pattern is transferred. To ensure this adhesion, the coverslip is aminosilinated to covalently bind it to the PAA hydrogel as it polymerizes.

Aminosilanization of the coverslip occurs through the following process, which is similar to those which has been described previously (Aratyn-Schaus et al., 2010; Kraning-Rush et al., 2012a; Kraning-Rush et al., 2012b; Rajagopalan et al., 2004). Large, 35mm coverslips are first cleaned through sonication in ethanol and then water. After sonication, the coverslip is plasma cleaned for 1 min. The plasma cleaned coverslip is then coated with a thin layer of 5% 3-APTMS using a glass pipette. After approximately 5 min, the solution on the coverslip will have dried and can be rinsed off thoroughly using DI water. Then, the coverslips are immersed in a 0.25% solution of glutaric dialdehyde for 30 min. After incubation, the coverslips are rinsed again with DI water and dried for use. They can also be stored in DI water for 2-3 weeks until use.
2.6 Transferring Microcontact Printed Patterns to PAA Hydrogels

Patterned coverslips are transferred to PAA hydrogels as they polymerize. PAA is a highly elastic, nonfouling material which has been used for other CTF techniques previously (Dembo and Wang, 1999). The Young’s modulus of PAA can also be altered to suit the needs of the experiment to demonstrate differences between soft and stiff material or to mimic the stiffness of a particular tissue over a wide range.

As PAA polymerizes, the protein forms covalent bonds to the NHS-ester found within the PAA and adheres to the gel. This allows for the protein to remain adhered to the gel during the duration of the experiment and to prevent it from being removed from the gel itself. PAA is a non-fouling material as well, which means that protein will not adhere to it.

The following procedure describes how the PAA hydrogel was created. In the experiments, there were several different solutions of PAA hydrogels used. The volumes of the solutions for each type of hydrogel are listed in Table 2.6.1 for a 5ml volume of hydrogel. For each hydrogel solution, the 40% acrylamide, 2% bis-acrylamide, and DI water were all gently mixed together first. The solution was then degassed for 15 min to remove as much air as possible. After degassing, 10 µL of TEMED was added to the solution. To prevent the hydrolysis of the acrylic acid N-hydroxysuccinimide ester (NHS-ester), 2N hydrochloric acid was added to adjust the pH of the solution to between 7.0-7.2. After this, 50 µL of NHS-ester was added at a concentration of 1 mg/mL, followed by 25 µL of 100 mg/mL of ammonium persulfate (APS). The solution is then gently inverted to facilitate mixing without introducing additional air. Finally, the solution is
pipetted on top of the activated coverslip. The volume can vary from 20 to 300 µl depending on the application. All that is required is that the gel be thick enough that the cell cannot sense the glass coverslip underneath, which requires a thickness of approximately 40 µm (Buxboim et al., 2010).

The patterned coverslip is then placed onto the PAA hydrogel with the pattern side facing the PAA gel solution. After 90 min, the coverslip can be removed by disassembling the gel device and removing the top coverslip. During the polymerization time, the gel should be placed in a petri dish covered with aluminum foil to avoid photobleaching the fluorescently labeled protein. After removal of the coverslip, the gel is incubated for 45 min with a 4% sterile, filtered bovine serum albumen (BSA) solution in 1x PBS to ensure that the PAA remains non-fouling.
2.7 Measuring the Mechanical Properties of Polyacrylamide Gels

PAA hydrogels that were fabricated were mechanically tested in order to check their physical properties. The bulk properties of the hydrogels were tested to obtain the Poisson’s ratio of the gel (ν) and the shear modulus of the gel (G). Uniaxial stretching of bulk samples of the gels and measuring the change in lateral dimensions provided the ν, while rheometry provided the storage (G') and loss (G'') moduli. Since the gels were found to be predominantly elastic (G'' was much smaller than G'), the following equation was applied to calculate the Young’s modulus (E) of the gel:

\[ E = 2G'(1 + \nu) \]
2.8 Procedure for Labeling Paxilling

Mouse embryonic fibroblasts (MEFs) were fixed and labeled using an anti-paxillin antibody to demonstrate that the cells were only adhering to the micropatterned adhesion points. Paxillin is often found to be associated with FA complexes and therefore is one good marker of the development of FAs.

MEFs were fixed by using a 4% solution of formaldehyde in PBS at 4°C for 15 min. The cells were then permeabilized using 0.5% TRX-100 in PBS at room temperature for 15 min. After this, the cells were incubated with 2% filtered BSA for 45 min as a blocking protein. Anti-paxillin monoclonal antibody (Y113) was diluted 1:200 in PBS with 1% BSA and then incubated with the sample overnight at 4°C. The sample was incubated for 1 hr with an Alexa Fluor 555 labelled donkey – anti-rabbit IGG (Abcam, 2mg/mL) diluted to a working concentration of 1:500. After this, fluorescent images of the cells were taken.
Figure 2.2.1 Patterning of Fluorescent Dots.

(A) Fluorescent fibronectin solution was adsorbed onto a plasma treated PDMS stamp.

(B) After adsorption and drying, the stamp was placed into contact with a plasma treated glass coverslip for 15 min. (C) The coverslip is placed onto the PAA pre-polymer solution. (D) After polymerization, the coverslip can be removed and the surface passivated where there has been no protein adhesion. (E) Cells can then be placed on the surface where they will exert CTFs. Adapted and reprinted from (Polio et al., 2012) with permission.
Figure 2.3.1 An Example of a Moiré Pattern.

As can be seen, there is poor alignment of the dots in the lower right hand corner; however, due to a small difference in the horizontal and vertical spacing in the shapes, better alignment is achieved in the upper left hand corner of the image. As long as the axes are well aligned and located at least 2.5 μm, the patterns are able to be used for determining CTFs.
Figure 2.3.2. Dual Patterning of Fluorescent Dots.

(A) Fluorescent gelatin solution was adsorbed onto a plasma treated PDMS stamp. (B) After adsorption and drying, the stamp was placed into contact with a plasma treated glass coverslip for 15 min. (C) Before removal of the stamp, the stamp and coverslip are placed onto a surface with an alignment marker, which will align with one edge of the pattern on the stamp. (D) The stamp is removed and a second placed onto the coverslip, aligning it with the previous alignment marker. (E) The coverslip is placed onto the PAA pre-polymer solution. (F) After polymerization, the coverslip can be removed.
Figure 2.4.1 Removal of Fluorescent Pattern.

(A) Fluorescent fibronectin is adsorbed onto plasma treated PDMS stamp. (B) The PDMS stamp is then placed into contact with a plasma treated glass coverslip. (C) A gluteraldehyde treated PDMS stamp with the desired features were then placed in contact with the coverslip to remove protein in unwanted areas. (D) The coverslip was placed onto the PAA pre-polymer solution with NHS-ester and (E) then removed once the gel had polymerized.
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<td>175 µL</td>
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<tr>
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<tr>
<td>APS</td>
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<td>25 µL</td>
<td>25 µL</td>
<td>25 µL</td>
</tr>
</tbody>
</table>

Table 2.6.1. Hydrogel Recipes.

This table shows the hydrogel recipes for the various gels used in the experiments.

Increasing concentrations of bis-acrylamide increase the stiffness of the hydrogel.
CHAPTER 3: IMAGING AND CTF CALCULATIONS

3.1 CTF Calculations

The CTFs of cells can be calculated through the use of a simple formula:

\[ F = \frac{\pi E au}{(1+v)(2-v)} \]

Where \( F \) is the force exerted by the cell, \( E \) is the Young’s modulus, \( a \) is the radius of the adhesion, \( u \) is the displacement vector, and \( v \) is the Poisson ratio of the material. This equation was derived to demonstrate the effect of force on a semi-infinite elastomeric material (Maloney et al., 2008). This equation assumes that the thickness of the elastic substratum is semi-infinite and that the loading is tangential to the surface of the hydrogel.
3.2 Imaging of CTFs

After cells are placed onto the surface of the PAA gel, they can be imaged using fluorescence to observe the labeled protein. In the studies conducted for my research, Alexa Fluor 488, Alexa Fluor 633, and Oregon Orange were used as dyes for the proteins although in practice, any readily visible fluorescent dye could be used as long as there were no other interfering fluorophores in the sample or cross-talk between fluorescent channels. Images were taken using either a 40x water lens, 60x oil lens, or 60x water lens mounted on an Olympus IX81 microscope with a Hamamatsu Orca R32 camera.

Generally, cells were imaged 12 hrs after plating to allow them enough time to adhere, though for certain experiments, this initial seeding time could be significantly shortened to accommodate different experimental parameters. Imaging the cells only required one image of the fluorescent patterns; however, it is encouraged to image the cells using brightfield microscopy as well so that the appearance cells on the substrates can be documented as well.
3.3 Cell Traction Image Analysis

Image analysis of the CTFs was carried out using MATLAB (Mathworks). Fluorescent images of the patterns, once captured provide a final, deformed pattern of the dots. Since we know the pattern of an undeformed pattern, it is then possible to attempt to extrapolate the initial positions of the dots knowing that not all of them have been deformed. This strategy has been used to calculate the deformations of mPADs. It is viable so long as there are known undeformed portions of the pattern from which to work.

During the course of this project, there have been two programs that have been developed to calculate CTFs, which employ different strategies. The first program version attempted to calculate deformations by the selection of the undeformed positions on the fluorescent image. First, the four corners of a rectangular shape were selected to highlight undeformed positions. Next, the user would input the number of points between the dots on each axis. The program could then calculate the spacing of the adhesion points. This was useful in that in an automated strategy, the number of adhesion points could change if there was a missing dot or there were different spacings depending on the axes and magnification. A grid was generated based on the selection of the dots. First, a line was drawn across the image from the top corners of the image to create a line with dots at set intervals. Next, this was repeated by drawing lines across the shape at set intervals to populate the rest of the shape with markers.

To determine the displacement of the dots from their initial positions, a tracking algorithm was applied to find the dots and how they had moved from their initial to final
position. This aspect of the program was borrowed from Blair and Dufresne (http://physics.georgetown.edu/matlab/) and performed well in identifying the displacements in a timely manner.

It was believed that the selection of points was much too slow in calculating the tractions and the program needed to be automated. To calculate the grid and spacing of the dots in the new program, the radon transform of the image was taken to determine the dots' spacing and the degree to which the image was tilted. Refinements in how the dots were localized and tracked were also made to improve localization of the dots. After determining the angles at which the grid is tilted, grid lines are drawn along each row of dots. At the intersections of these points, the initial position of the dot would be placed is found. The code for this version of the program is included in Appendix 1.
CHAPTER 4: SINGLE PATTERNING RESULTS

4.1 Cell Adhesion

Our novel TFM that we propose will seek to determine the deformation of a PAA substrate in response to cells pulling on distinct adhesion points. This technique will simplify the measurement of CTFs as cells will only adhere to and deform these regularized fiducial markers. Given a priori knowledge of the patterns through the use of microcontact printing and tracking the deformation with fluorescently labeled adhesion points, we can calculate the traction forces using a linear equation. Previous techniques required the fabrication of highly precise pillar arrays or complex mathematics to achieve similar results. For this system to function effectively, FAs formed by the cells need to be limited to the micropattern itself. Therefore, we needed to demonstrate that the proposed PAA substrate was non-fouling and would not allow the adhesion of cells.

In order to demonstrate that cells would exclusively adhere to the micropatterns and not to the unpatterned PAA hydrogel, the PAA hydrogel surface was coated in a small area with fluorescently labeled Fn (Polio et al., 2012). Protein that had been adsorbed to a glass coverslip in a circular shape was transferred to a PAA hydrogel, then incubated with BSA. Figure 4.1.1 shows how the fluorescent protein (Figure 4.1.1A) confined the NIH 3T3 fibroblasts, which were imaged in brightfield (Figure 4.1.1B), to the patterned area. There were no cells found to be extending into areas without patterned fluorescent protein, which is represented by a yellow line on the border of the fluorescent pattern in both the fluorescent and brightfield images. This also demonstrated our BSA incubation was effective in preventing cell and other nonspecific protein adhesion on the
hydrogel. By quenching the remaining NHS-ester groups that were not functionally adhered to Fn, cells could only adhere to the initial area that was micropatterned.
4.2 Staining Cells on Dots

As stated previously, demonstrating that the FAs of cells would only be forming on the desired adhesion points was of paramount importance. As cells exert force on their surroundings, it is important to demonstrate that the cells are only pulling on these adhesion points exclusively. With mPAMs, this is also a known issue as cells can adhere to the sides of the posts will cause deformations that are not able to be accurately transformed into forces. If this is not true, then there would be significant consequences as the technique depends on this in order that the simplified equation translating deformation into force can be applied.

Demonstrations of the exclusivity of cell adhesions on micropatterned dots was done by labeling paxillin, a protein associated with FAs on Fn. It was of concern that although the protein was transferred to isolated adhesion points that the cell would still be mechanically interacting with the PAA hydrogel due to the fact that cells are continually remodeling their surrounding environment through the production of ECM in the presence of serum. This was a concern as it is known that micropatterning is not consistent over the whole patterned area of the glass coverslip and at times, protein could be found between the adhesion points.

To address the concern that in the well patterned areas, cells were adhering only to the micropattern, we fluorescently labelled the FA protein paxillin with an antibody. Paxillin is generally found localized to the FA complex within the cell itself as it is part of the mechanosensing machinery (Katz et al., 2000; Yamada and Geiger, 1997). Although paxillin has been found to extend in areas beyond the point of adhesion
previously, labeling the FAs would help to visualize their location and the relative
direction of pulling. Therefore one would expect the fluorescence to be co-located to
those positions that are close to the patterned points on the PAA gel.

Figure 4.2.1 shows an image of mouse embryonic fibroblasts (MEFs) on an
evenly micropatterned surface. It can be seen that the paxillin is localized to punctate
points that are spread throughout the cell. As the cell is not limited to adhere to any
particular area, the FAs are perpendicular to the protrusions of the cell. This is in contrast
to the panels shown in Figure 4.2.2. The fixed MEFs on a patterned PAA surface of the
same PAA gel have much of the strongest staining localized to and around the adhesion
points. It can be seen from the images that cells on the patterned PAA form punctate FAs
close to the micropatterned areas and extend beyond them as well due to the fact that
paxillin is associated with the intracellular machinery of the FA and may not be located
exactly at the adhesion points.
4.3 Gel Properties

Next, we needed to determine the material properties of the hydrogels we were using for the experiment. In order to convert deformations into traction forces, we needed to find the bulk properties of the hydrogels, which can vary depending on the formulation and material properties. By measuring the shear modulus and Poisson’s ratio of the hydrogel, we could produce the Young’s modulus as well as any other desired property of the hydrogels. We also needed to demonstrate that in the regime in which the cells were deforming the hydrogel that PAA would be appropriate as these deformations would be elastic in nature.

Prior to the experiment, the polymerized of the samples had been left in PBS overnight at 4°C. The storage and loss moduli of three of the hydrogels were measured using an AR2000 parallel plate rheometer at 37°C to replicate the experimental conditions. From this data, the shear moduli were converted to Young’s moduli over a low frequency sweep of 0.01-10 Hz (Table 4.3.1). It was found that the hydrogels were primarily elastic and only a small contribution of the stiffness in this frequency was due to the viscosity of the gel, as measured by the loss modulus. Increasing bis-acrylamide concentration resulted in increasing the Young’s modulus, as predicted based on previous work. From the vertical stretching experiments on 0.07% bis-acrylamide hydrogels, it was found that the Poisson’s ratio of the hydrogels was 0.445, which is in agreement with other experiments on PAA hydrogels.
4.4 Program Results

In order to make this approach accessible to labs with basic engineering tools, we needed to develop a robust image processing algorithm in a common programming language. We therefore decided to create a MATLAB program that people with basic knowledge of the language could use to convert displacements.

We used the first iteration of our MATLAB script to convert the fluorescence images of the fiducial dot markers into displacement and traction vectors. The first test was to use this program to analyze the inherent error associated with our technique for patterning the gel, since patterning errors may lead to apparent dot displacements even in the absence of applied force. Therefore, it was necessary to find a threshold value below which CTFs would be found to be negligible.

A sample image of fluorescent dots on a PAA gel is shown, with apparent dot displacement vectors added for each dot that were determined from the analysis program (Figure 4.4.1). No cells were present on the gel for these measurements. The cumulative distribution function (CDF) of all 450 displacement vectors analyzed in this field of view is shown in Fig. 4.4.1B, where the average displacement was found to be $0.10 \pm 0.05 \mu m$. From these data, we can determine that a threshold displacement value of $0.3225 \mu m$, or 3 pixel widths for the given camera at 60x magnification. The corresponding traction force of $0.98 \text{nN}$ with this gel stiffness, would eliminate close most of background vectors. Consequently, we adopted this value and assigned all displacements $\leq 0.3225 \mu m$ to a value of 0 in future cell studies in order to better represent the CTFs exerted on the surface.
In order to provide perspective on the theoretical limits of the technique, it is also shown how the size of the dots would affect the measurable displacement given the average displacement of dots from this technique (Figure 4.4.2). Larger dots and stiffer substrates will increase the amount of force necessary to determine tractions.
4.5 Cell Traction Force Results

Images of cells were acquired to demonstrate the CTFs exerted on the substrate. A fluorescence image of the Alexa Fluor 488 labeled Fn pattern (Fig. 4.5.1A) is shown overlaid with the bright-field image of a fibroblast cell after 24 h in culture on the patterned PAA gel (Fig. 4.5.1B). The figure shows the flow of data in the analysis. First, the MATLAB program identifies the locations of the dots in the fluorescence image using the algorithm mentioned in Chapter 3.2. The user then selects the corners of the image and the number of dots between the selections. From these selections, the relaxed positions can be found to determine the patterned dots' initial positions. The displacement vector is then calculated (colored by magnitude) for each dot, selecting the most likely candidate for its initial position based on the final position (Fig. 4.5.1C). The CDF of all displacement vectors in the field of view is shown for an unthresholded images for both the area with the cell (blue) and a micropatterned area without cells (red; from Fig. 4.5.1C) (D). After thresholding, all dots with displacement vectors with magnitudes >0.3225
found that the sum of forces was $4.03 \pm 2.59$ nN. Another important mechanical concept is the balance of moments. The moments should balance such that the ratio of $M_{yx}/M_{xy}$ should have a ratio of 1. For the same cells, it was found that the balance was $1.01 \pm 0.10$. 
4.6 Single and Multi Cell Shape

With this technique one goal is to give the users as much control as possible over the many variables that will affect the exertion of CTFs onto the substrate. One of the ways in which cells are stimulated to respond via CTFs is through cell spreading. Control over the CSK has a large influence on the amount of traction and spreading of the cell (Han et al., 2012). In order to exert shape and cell spreading of cells on our features, we created islands of adhesive dots to which the cells could adhere and spread over.

Isolated sections of patterned proteins were created to confine single and multiple cell groups to defined areas. CTFs were then calculated for the cells on these substrates. Notably, in the case where cells were mostly covering the surface of the hydrogel on these shapes, there were few traction forces underneath confluent layers of cells. The CTFs were localized to the edges of the patterns in the square shape (Fig. 4.5.1). This suggests that within the cell cluster the contractile forces were primarily transmitted via cell–cell coupling, whereas at the edges of the cluster, in the absence of cell–cell coupling, force transmission is primarily via cell–substrate interactions. Further experiments would be needed to confirm this; however, it has been shown that disruption of the cell-cell interactions in similar circumstances leads to an increase in CTFs on the substrate (Maruthamuthu et al., 2011). This phenomenon can be seen on patterned islands ranging from 15 x 15 μm² to 50 x 50 μm² (Polio et al., 2014; Polio and Smith, 2014).
4.7 Comparison to Previous Techniques

In order to demonstrate how the technique compares with previous traction microscopy techniques, we embedded beads into a PAA hydrogel, which was patterned with fluorescent FN. In this experiment, we could directly compare displacements and traction forces using the same cell using code developed by Butler et al. (Butler et al., 2002). Specifically, unconstrained FTTC was used to determine the displacements and CTFs using the embedded bead technique. Figure 4.6.1 shows a comparison of the displacements while Figure 4.6.2 shows a comparison of the traction forces (Polio et al., 2014).

Despite only being able to adhere to punctate points, the CTFs tended to propagate much further than the dots themselves. In fact, the forces tend to propagate beyond the cell itself as well. The technique also tends to underestimate the displacement of the adhesion points compared to those determined by the technique we presented. It is difficult to compare the traction forces themselves as the methods produce different results. MTM produces vectorized point forces while embedding beads within a gel produces forces that are interpreted using a field and thus a force per unit area measurement. However, what is apparent is that it is an underestimation of these forces as the displacements at or near the points of the dot appear to underestimate the displacement of the dot itself as well.

The difference between the two techniques is in large part due to the methods used to calculate the displacements and limitations of the techniques. MTM directly determines the deformations exerted on the substrate by the cells through tracking the
centroid of the displaced adhesion point. This is in contrast to the way other techniques using randomly dispersed beads. The beads themselves are not tracked, but the regions are cross-correlated in order to find the displacements. Tracking the displacements of the markers is not possible as it is in MTM as the combinatorics involved in determining the potential candidate displacements is much lower in MTM than using high densities of beads, which may have many initial candidate positions.

The cross-correlation of regions of high densities of beads must be used to determine their displacements. The major, known drawback of this technique is that as the mesh size used to cross-correlate decreases, fewer data points are able to be mapped, which is a significant problem when trying to determine the displacements due to point forces (Sabass et al., 2008). Due to Nyquist sampling limitations, FTTC and BEM will underestimate smaller point forces as the spatial resolution of each subdivision is not sufficiently high enough to determine tractions. Techniques such as TRPF have improved upon this technique; however, this method depends on the appropriate localization of distinct FAs through patterning or labeling, which can present an additional challenge.
4.8 Summary and Brief Discussion of the Results

In this chapter, it was demonstrated that MTM is a novel tool for determining CTFs in a unique manner that is amenable to being used with different types of soft biomaterials. By using an indirect patterning method, we are able to transfer micrometer-sized dots from a microfabricated stamp to a plasma treated glass coverslip with a high degree of precision. This type of patterning confers several benefits onto the technique. First, we have an \textit{a priori} knowledge of the adhesion points, which would allow us to acquire real time traction forces based on the previous knowledge of the pattern, similar to mPADs (Tan et al., 2002). Secondly, it allows us to manipulate the patterns much more easily.

By patterning onto glass, we are able to utilize techniques for micropatterning small, precise features with a variety of different proteins. A different adaptation of this technique has been used to pattern a wide variety of ECM proteins including laminin and collagen I (Tang et al., 2012). In addition, proteins important for cell-cell contacts, such as cadherins and ICAMs have been micropatterned as well, thus opening the technique to further possibilities (Borghi et al., 2010; Chopra et al., 2012; Shen et al., 2008b). It is important that a technique that is interested in delving into the nuances of traction forces be able to have a variety of options when it comes to patterning as it has been found that by varying ECM composition, CTFs can change significantly (Gershlak et al., 2013).

This technique also gives us control over not only the proteins that are able to be micropatterned, but the shape of the micropatterns by using pattern removal to limit the area to which cells were able to adhere. This allows for the initial patterning to be
performed in a similar manner each time without the worry of having to iteratively alter the photomask in order to achieve the desired results, a process which takes a significant amount of time and resources to perform as alterations to the photomask and substrate are expensive.

We also demonstrated through multiple techniques that the CTFs are localized to the patterned adhesion points and that the cells are not mechanically interacting with the hydrogel around the adhesion points. This is important as this provides a justification for the assumptions that we must make in order to calculate CTFs. This does not negate the possibility that cells could interact with the surrounding hydrogel in other ways, but as for determining traction forces, this is an appropriate assumption (Chopra et al., 2014).

Through basic force balancing principles and comparison to another TFM technique, we further demonstrated that the assumptions made are valid mechanically and within reason for the forces being determined. This provides evidence that this technique is mechanically sound and suitable for the calculations of CTFs. This is an important validation of the technique as sound mechanics should serve as the basis for this and other CTF techniques.
Figure 4.1.1 Cells Confined to Fibronectin Pattern.

A small drop of fluorescently labeled fibronectin was used to adsorb a circular region of fibronectin on a glass coverslip that was then transferred to the surface of a PAA gel containing NHS-ester during the polymerization phase. Fibroblast cells were then seeded onto the PAA gel and rinsed after 1 h to remove non-adherent cells. After 24 h of cell culture, both fluorescence (A) and bright-field images (B) of the interface of the fibronectin-coated circle and the non-passivated region of the PAA were acquired. The
line in (B) represents the edge of the fibronectin island in order to determine cell features that extend beyond the pattern. Reprinted from (Polio et al., 2012) with permission.

Figure 4.2.1 Evenly Coated Surface with Paxillin Staining.
Mouse embryonic fibroblasts adhering to areas of evenly coated fibronectin (red) were stained with an anti-paxillin antibody (green). The paxillin is generally arranged in punctate areas underneath the cell and is associated with focal adhesions. Scale bar is 20 μm.
Figure 4.2.2 Paxillin Stained Cells with Traction Forces.

Cell traction forces (A, D, G, and J) were measured for fixed cells that had been labeled with anti-paxillin antibody (green; B, E, H, and M). The images were overlaid onto one another to show the correlation between the fluorescently labeled fibronectin (red; C, F, I, L), which demonstrates that the cells are exclusively adhering to the fibronectin pattern. The scale arrow in the upper left hand corner (A, D, G, and J) and maximum force on the color bar represent 35nN. Scale bars represent 20 μm. Reprinted from (Polio et al., 2014) with permission from the Centre National de la Recherche Scientifique (CNRS) and The Royal Society of Chemistry (RSC).
<table>
<thead>
<tr>
<th>Bis-Acrylimide (%)</th>
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<tr>
<td>Young’s Modulus (kPa)</td>
<td>0.57</td>
<td>3.66</td>
<td>13.62</td>
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</tbody>
</table>

Table 4.3.1. Stiffness Measurements with Variations of Bis-Acrylamide.

Stiffness measurements of the storage moduli of different PAA hydrogels were converted to Young’s moduli.
Figure 4.4.1. Fluorescent Fibronectin Pattern Without Cells.

A patterned PAA gel was imaged under water and processed using the custom MATLAB code to generate apparent displacement vectors for each of the patterned dots. Displacement vectors are shown scaled by a factor of 10 times their actual length, corresponding to the color-coded displacement scale in μm (A). A cumulative frequency plot of all 450 dots that were analyzed in this field of view is shown (B), where the 25th, 50th and 75th percentile dots reside at 0.05, 0.094 and 0.12 μm, respectively. A histogram of the data was inset to demonstrate the distribution of the displacement vectors. Reprinted from (Polio et al., 2012) with permission.
Figure 4.1.2 Minimum Detectable Traction.

The minimum detectable tractions for 0.094 μm displacements for gels of Young’s moduli ranging from 0.5 kPa to 50 kPa for micropatterned dots with diameters of 1 μm (blue) and 2 μm (red).
Figure 4.5.1 Example of a Cell on a Micropatterned Substrate

(A) An image of a fluorescently labeled fibronectin pattern is (B) overlaid onto a bright-field image of an NIH 3T3 fibroblast cell. (C) The actual displacement vectors of the dot pattern by the cell are shown as determined by the analysis program. The colors of the vectors are correlated to the magnitude of the displacements. (D) A cumulative distribution plot of the cell in the image (blue) is shown before thresholding the
displacements as compared to an image without cells (red). After thresholding displacements less than 0.3325 µm in length, the remaining vectors were converted into tractions vectors and are shown without (E) and with (F) the cell on the pattern. Reprinted from (Polio et al., 2012) with permission.
Figure 4.5.1 Example of a Processed Square Patterned Image.

(A) A fluorescent Fn image is taken of the deformed points. (B) The radon transform of the image shows two prominent bands at 791 and 1691, which are the angles of tilt of the two axes of interest. (C) A grid is drawn on top of the image. The intercepts mark the original positions of the dots. (D) By determining the displacement of the dots from the original positions, the traction forces are calculated in nN. Reprinted from (Polio et al., 2014) with permission from CNRS and RSC.
Figure 4.6.1. Displacements of MTM vs. FTTC

(A) The displacements of MTM vs. FTTC demonstrate a fundamental discrepancy in the techniques. The displacement of the patterned dots which have arrows generating from
the adhesion points (green) does not match with the displacements calculated by the FTTC calculation, represented by arrows generating from outside the green dots. FTTC regularizes the array of displacements in order to simplify CTF calculations. The black line represents the cell boundary. The blue box is a sample area which was enhanced (B) to give a better visual representation of the discrepancies. The colorbar represents the displacement in μm.
Figure 4.6.2 Comparison of Traction Forces.

(A) Two cells were imaged on the surface of a gel that had been patterned with fluorescent fibronectin as well as contained 1 μm beads. (B) Cell traction forces were calculated using MTM and the cells were outlined in white. The color scale bar represents cell tractions in nN. (C) Cell traction forces were calculated using FTTC on the same PAA gel after the cells were removed. The color scalebar represents forces in Pa. A zoomed in region for comparison is shown to demonstrate the adhesion points for both (D) MTM and (E) FTTC. Scale bars represent 5 μm. Reprinted from (Polio et al., 2014) with permission from CNRS and RSC.
CHAPTER 5: DUAL PATTERNING TRACTION RESULTS

5.1 Dual Patterning Technique

Previous experiments on that studied mechanobiology using TFM techniques have generally been conducted on single ligand substrates. However in order to replicate conditions similar to those found in \textit{in vivo} environments which contain multiple ECM components, we wanted to explore the use of multiple ligands and to determine the contribution of each ligand to the overall traction of the cell. This would be the first system to calculate CTFs on a substrate with multiple, spatially distinct proteins. By having them be spatially distinct, it allows for the determination of forces being exerted on each protein individually. This type of study is important to mechanobiology as not all the ways cells physically interact with their environments that affect CTFs can be observed through mechanics (Chopra et al., 2012). Cross-talk mechanisms that result in the changing of forces may also change which ligands cells are interacting with as well (Chopra et al., 2014). This is in contrast to techniques that would mix ligands together to determine traction forces as it is difficult to determine how each matrix component is contributing to the change in traction (Gershak et al., 2013).

We developed an approach that used Moiré patterning to simplify the alignment of multiple micropatterned ligands on a glass substrate (Figs. 2.3.1 and 2.3.2). With this approach, only alignment along one axis is required, thus greatly simplifying the generation of appropriately spaced dual patterns. Figure 5.1.1B shows an image of a substrate that has been patterned with multiple ligands. Alignment using the template underneath the glass allowed for removal of the first stamp, while keeping the user aware
of its original position. This is very desirable as similar alignment techniques are often complex and require the development of custom equipment. By aligning the second stamp such that features are parallel relative to the first, it alleviates errors that may have been generated by aligning both to a fixed template where variations in one pattern’s position would result in variations relative to the second pattern. The alignment can be done by hand without the use of any other complex mechanical devices. Therefore, labs can adopt this technique without significant investment in additional resources.
5.2 Dual Patterning Forces

CTFs of individual and groups of cells were able to be calculated on dual patterned substrates (Figs 5.1.1C and 5.1.1D). In this way we could determine the contributions of the cells to each traction pattern. Also, we could determine how the presence of both gelatin and Fn would alter the traction forces exerted by the cell. For the dual patterned surfaces, we were able to find the traction forces on each of the micropatterned dots surfaces by analyzing each fluorescent image separately. Deformed micropatterned dots located close to each other did not affect the deformation of neighboring points in all but the most extreme cases where center-to-center distances were less than 2 μm. Interestingly, we were able to show (Fig. 5.2.1) that in the presence of gelatin, CTFs were almost exclusively limited to the gelatin pattern, while traction applied to Fn dots was limited or absent. Moreover, the traction forces on gels patterned with Fn alone were much greater than on the dual patterned gelatin–Fn hydrogels, despite the fact that the dual patterned substrates had more protein, thus allowing for greater traction forces.
5.3 Summary and Brief Discussion of the Results

In this experiment, we were able to determine CTFs on PAA hydrogels that had been patterned with multiple, distinct proteins. The ability to pattern and study cell-ECM and potentially cell-cell interactions on PAA using this technique provides an interesting opportunity. We were able to continue the development of previous research to establish this technique as a complementary technique, allowing us to study CTFs as they interact with other ECM proteins. As cells inhabit complex environments in vivo, it is important that we be able to determine how interactions with multiple ligands affect cellular behavior.

Previously, it was shown that integrins have the ability interact in synergistic ways that causes CTFs and cell spreading to be altered (Byfield et al., 2009; Lin et al., 2013). In fact, previous techniques have also attempted to understand how cells interact with each other by calculating the forces with which cells interact through the use of force balancing (Maruthamuthu et al., 2011; Nelson et al., 2007). Through the use of this technique, it is possible to directly measure the cells’ interactions at punctate points with a substrate that has mixed ligands of individual and groups of cells without having to rely on finding cells in an appropriate geometric arrangement to easily calculate the tractions.

Due to the indirect patterning onto glass and then transferring the pattern onto the PAA hydrogel, this technique is able to be modified to accommodate multiple proteins, such as cadherins or other cell-cell or cell-ECM proteins. This would allow for the study of cross-talk at the FAs and lead to a deeper understanding of how ECM composition as well as cell-cell interactions leads to changes in CTFs.
Figure 5.1.1 Dual Patterned Traction Forces.

(A) A dual pattern of Alexa Fluor 633 labeled fibronectin (red) and gelatin (green) was patterned using the dual patterning technique described and cells are seen to be adhering to the dots. (B) The dual pattern is pictured with only fibronectin and gelatin. Traction forces for (C) fibronectin and (D) gelatin were able to be separated due to the difference in fluorescent labeling. Scale bars are 20 \( \mu \text{m} \). The colorbar scale is in nN. Reprinted from (Polio et al., 2014) with permission from CNRS and RSC.
Figure 5.1.2 Expected Cumulative Distribution of Dual Patterns.

The ECDF of tractions corresponding to Fn only (blue; n = 5), Fn dual patterned (red; n = 4), and dual patterned gelatin (green; n = 4). Reprinted from (Polio et al., 2014) with permission from CNRS and RSC.
CHAPTER 6: DISCUSSION AND FUTURE WORKS

6.1 Discussion of Technique Development and Advantages

The intent of this project was to demonstrate the development of a new, unique TFM technique that would greatly simplify the process of TFM. MTM provides a powerful, open platform to measure CTFs on multiple surfaces with a wide array of proteins. The development of this technique provided an important tool for probing the mechanical nature of cell-ECM interactions and, in the future, cell-cell interactions that have not been able to be studied in this manner previously.

The first aim of the project was to develop an indirect patterning technique that could measure CTFs without cell removal to provide information of CTFs during the course of the experiment. To demonstrate that it was a reliable, mechanically sound technique, we showed that PAA hydrogels were a prime, nonfouling medium for the determination of CTFs. Not only would cells not mechanically interact with bulk PAA hydrogels that had been passivated, but also the cells would not mechanically interact through FAs with areas between the micropatterned dots. Although this does not preclude the ability for cells to sense the properties of the hydrogels in other manners, it provides a sound mechanical basis for further assumptions to be made (Chopra et al., 2014; Trappmann et al., 2012).

The ability to pattern onto PAA also allows for the stiffness of the substrate to be altered to stiffnesses over several orders of magnitude. Altering the material stiffness or its perceived stiffness is fundamentally necessary to any CTF technique as it has been found that not only does the stiffness of the material have a significant role in
determining the fate of stem cells (Engler et al., 2006; Nava et al., 2012; Yeung et al., 2005), but also in the functioning of mature cells (Levental et al., 2007; Saha et al., 2008). In order to replicate the mechanical environment of cells to match *in vivo* stiffnesses, the stiffness of the underlying matrix requires modulation to provide an accurate *in vitro* representation of the appropriate cell phenotype (Ulrich et al., 2009).

This technique has the added benefit that it measures CTFs as discrete points, which are reminiscent of how cells apply forces. The FAs of cells, the major force producing mechanism by which the CSK interacts with the cell’s surrounding environment has been shown to exert force in a discrete manner in this study as well as others through staining of FA-associated proteins (Balaban et al., 2001; Oakes et al., 2012). These other techniques have measured CTFs as a field out of necessity as without distinct labeling of FAs or micropatterning to confine FAs to defined areas, distinguishing the forces exerted by an individual FA would be a difficult computational task and not all cells are amenable to transfection to fluorescently label FA proteins (Maruthamuthu et al., 2011; Sabass et al., 2008; Schwarz et al., 2002; Stricker et al., 2010). Determining the forces exerted at individual FAs as compared to the cell overall is an important as individual FA dynamics could be of great interest in studying time-varying cell behavior, such as in the case of cellular reorientation or in the mapping of cytoskeletal prestress (Canovic et al., 2013). Therefore, it is befitting that in developing a new approach, we would develop a technique that was consistent with the measurement of traction forces as point forces instead of as a field. This provides a *de facto* pre-labeling of the FAs themselves as well in that cells are limited to forming them at these
discrete points on the substrate.

This technique also provides other important technical advantages over previous techniques that make it amenable to being adopted. Since the cells are on a nonfouling PAA hydrogel, they will not remodel the space between the hydrogel dots over long time periods. This is in contrast to materials such as PDMS that do not have the ability to prevent adhesion due to the adsorption of protein over time. The ability to have covalently attached protein through the use of bifunctional crosslinkers allows for many different types of proteins to be used on other engineered hydrogels as well or in some cases, the protein can adhere to the hydrogel through nonspecific mechanisms (Chopra et al., 2014; Tang et al., 2012). The advantage of using this technique is that even on thin film materials or other configurations, there are currently applicable models to describe these scenarios (Maloney et al., 2008).

The analytical technique used in this study gives researchers access to real time information about CTFs without removal of the cell. Traction forces can be measured as soon as the images are acquired and in a short time period (often less than 30 seconds). The initial patterns can be inferred from the final positions of the fluorescent protein markers due to the fact that we have a priori knowledge of the initial pattern. Comparatively, TFM techniques such as FTTC that use beads dispersed within a hydrogel only provide data after the completion of the experiment and removal of the cell and a significant amount of computational resources and technical expertise in the mathematics required to analyze and interpret the data (Balaban et al., 2001; Schwarz et al., 2002). Our technique is available online for public consumption and undergoes
constant revision to keep the most updated version available (www.bu.edu/mml).

There are technical limitations to this technique that are important to be aware of. In choosing a CTF technique, it is critical to recognize that the appropriate technique must be applied for the situation. One drawback to this technique is that the technique is not amenable to understanding how CTFs change in 3D environments. Currently, there exist techniques that leverage the use of beads in 3D or 2D environments to determine how cells behave in a more contextually relevant environment (Baker and Chen, 2012; Legant et al., 2013) as cell behaviors, such as responses to drugs in a cancer assay, may be altered by the presentation of cells in a 3D environment (Hur et al., 2009; Maskarinec et al., 2009; Serebriiskii et al., 2008; Weaver et al., 2002; Weigelt et al., 2010). However, cells that tend to experience more 2D like environments, such as endothelial cells, or other cells which are interacting with flat surfaces, such as rolling leukocytes, are appropriate to study in a 2D context. This technique is intended to be a complementary technique to others and, as a tool, must be applied in the appropriate circumstances.

A few other technical limits to the technique also exist. It is apparent that with very large deflections, the linear equation that is applied to determine tractions is no longer valid. There are available timelapse videos which have been acquired that demonstrate this drawback. This can be compensated for by altering the dot density or substrate stiffness as the stiffer the substrate is, the less the cell will be able to deform it. However, there are treatments that can work if there are too many displaced features and the current stiffness and density are needed to be maintained, there are mathematical treatments based on the Green’s function that can be applied if the original positions of
the dots can be determined if desired; however, most techniques will experience similar difficulties in resolving strong forces being applied (Renault et al., 2002).
6.2 Discussion of Cell Traction Force Results

Another important advantage of this technique is that by patterning onto the glass coverslip, we are able to pattern a variety of proteins with a high degree of topographical control to regulate cell shape. Specifically, we have shown an ability to pattern gelatin and Fn simultaneously as well as pattern defined shapes.

In determining the CTFs, it was found that there is a cross-talk interaction between Fn and gelatin, which, when subjected to all reasonable research, has not yet been reported elsewhere. When placed on substrates with both gelatin and Fn, cells appear to alter their tractions by exerting an overall lower force, which was almost exclusively exerted on gelatin in this context. The main way in which the cells are interacting with gelatin and Fn is via the integrins presented in the FAs. It has been shown previously that the interactions between the integrins can in fact influence one another via cross-talk (Lin et al., 2013). Although the mechanisms by which the integrins are interacting are currently unknown, it is very intriguing that now we have a mechanism by which multiple proteins can be patterned to aid in searching for potential mechanisms through looking at the traction forces exerted in the context of different combinations of proteins now that we have the ability to discriminate tractions on each adhesion point.

By controlling cell shape, we also have provided a tool that can be used to determine cell tractions on various single and multicellular conditions. One benefit of indirectly patterning onto glass is that we are able to both pattern and remove protein in desired areas. This allows us to pattern features that would be very difficult to optimize
under normal patterning conditions, such as virtual islands of adhesion points with
enough separation from neighboring cells that there is no physical interaction between the
groups. Using traditional means, this would require some time to optimize as high aspect
ratio features are difficult to fabricate and stamp.

The forces being concentrated on the edge of the patterns themselves is very
characteristic of both individual and patterned groups of cells (Nelson et al., 2005; Rape
et al., 2010; Thery et al., 2006). Similar patterns of force exertion were observed in the
experiments conducted. One shortcoming of this technique is that due to the removal of
patterns, it can be difficult to determine the traction forces of cells which deformed most,
if not all of the points surrounding them. However, it is very likely that future work
would be able to resolve this problem through secondary patterning of inert markers, such
as fluorescently labeled BSA to assist in the location of the initial patterns of smaller
islands of dots.
6.3 Applications and Future Goals

The techniques that we have developed provide powerful and unique opportunities to study CTFs in contexts that have not been previously been experimentally feasible as well as in the pursuit of the study of more classical CTF studies as well. We are also hoping in the future to develop applications for this assay to provide clinically relevant information. In demonstration of the potential of these applications, we and others are currently working on and are able to propose a number of projects that have uniquely benefitted from the development of the techniques herein. These applications range from simple improvements to CTF calculations over current techniques to exploring novel questions about cell mechanics and cell-ECM interactions.

6.3.1 Extracellular Matrix Remodeling with Cell Traction Measurements

Due to the nature of cells being restricted to forming FAs onto the patterned protein itself, one experimental observation that can be observed is the remodeling of the ECM. It is known that ECM, such as Fn, undergoes dynamic remodeling when cells are placed onto substrates of various stiffness. Fn is unique in that the application of strain has been shown to result in the unfolding of the molecule itself (Bradshaw et al., 2012; Smith et al., 2007). As the rigidity of the substrate is increased, so too is the amount of matrix produced by the cells (Carraher and Schwarzbauer, 2013). It has also been observed that when remodeling ECM on stiffer surfaces, cells are placing more strain on the ECM itself (Antia et al., 2008). Therefore, determining the forces involved in how cells remodel and produce Fn matrix is of great importance to the study of mechanotransduction as a whole.
With control over adhesion ligand spacing and type, and the shape of the cells themselves, we are able to study how cells exert force on their surrounding environment as they remodel the substrate. Figure 6.3.1 demonstrates that cells in the context of our system are able to remodel the substrate. Fluorescently labeled Fn in a different color from the pattern was shown to be assembled by MEFs over time as they were observed on the substrate.

Observations of the forces related to ECM remodeling and understanding how this type of remodeling force changes with time is of interest in mechanobiology. Currently, there have not been studies that have shown a correlation of the strain of the matrix made with simultaneous measurements of CTFs being exerted on the substrate. By extending this to micropatterned islands, the amount of force as it relates to the island shape would allow the exploration of ECM remodeling in the context of shape control. There have been some studies that have used Forester resonant energy transfer (FRET) labeled Fn to observe how cells remodel their surrounding Fn matrix; however, there have been no apparent studies that have looked at the change in FRET ratio with the traction force of individual cells. Similar studies have been carried out by trying to understand this remodeling by groups of cells (Legant et al., 2012). Future studies that demonstrate the forces related to ECM remodeling in 2D will explore how these forces change in relation to the substrate stiffness as well as the time the cells are assembling the matrix.
6.3.2 Traction Forces in 2.5D Environments

Currently, our traction force system can only measure traction forces in 2D; however, it is obvious that many cells inhabit 3D environments. 3D CTFs make up an area of intense field of study that has had a number of recent advancements (Baker and Chen, 2012; Chang et al., 2013; Koch et al., 2012; Legant et al., 2013; Tekin et al., 2012). Also it is very apparent that cells in 3D can behave much differently than cells in 2D environments, specifically with regard to cell function (Dunn et al., 1989).

Some of the early ways in which 3D environments were presented to cells were in so called “2.5D” environments where cells would be sandwiched between two hydrogels (Beningo et al., 2004; Dunn et al., 1989). This type of study is physiologically relevant as many cells have basal and apical polarizations, such as epithelial cells, and some cell types, such as smooth muscle cells, are exposed to different surfaces due to the structure of tissues (Smiley-Jewell et al., 2002). Here we have a unique opportunity to present mixed ligand environments to the cells that could be more representative of what the cell is being subjected to in vivo.

Through the use of multiple fluorescently labeled proteins, we can pattern different proteins to present a polarized environment to the cells, such as one that could be observed by epithelial cells, which have basal and apical sides. By patterning one face with proteins such as cadherins and the other with various ECM proteins that comprise the matrix at the basal membrane, such as Fn, laminin, or collagen, we could determine how CTFs change with dimensionality and by presenting ligands on different surfaces of the cells. CTFs have been measured in this manner previously; however, it has been
restricted to only one type of ECM protein (Beningo et al., 2004). Preliminary experiments have demonstrated that this is feasible and a potentially powerful technique.

6.3.3 Current Uses and Adaptations of Micropattern Traction Microscopy

To demonstrate the ease of adaptability of this technique, we have been open in collaborating with many other investigators in order to aid in its more widespread adoption. Even without our assistance, there have even been published works that have adapted this set of techniques to suit their own experimental needs to explore other novel ideas.

Currently, we have used MTM as a novel technique to aid in the mapping of cytoskeletal prestress with a technique known as biomechanical imaging (Canovic et al., 2013). MTM is unique in that it can be used map subcellular CTFs at discrete points on a flexible substratum that can be stretched easily without the removal of cells using a relatively simple system for stretching (Figure 6.3.2). By applying a brief strain and the imaging the change in CTFs and the positions of intercellular markers, the cytoskeletal prestress can be determined in an offline analysis from this brief, 30 second test. The advantage of using MTM is that there is an ability to acquire tractions and prestresses over a large area of an individual cell, which is important as cells have been reported to have a wide range of variability in their stiffness (Guo et al., 2012; Park et al., 2005). The technique provides a prestress map of the cell itself, offering a new tool that can be used to determine the mechanical properties of individual cells.

The Lam Lab at Georgia Institute of Technology has adapted the technique we developed in order to pattern fluorescent dots of fibrinogen onto PAA surface that could
be used to measure the contraction of platelets (Figure 6.3.3). This technique served as a
great simplification of their previous technique, which required the capturing of
individual cells and measuring the applied forces using AFM (Lam et al., 2011). For this
group, patterning and measuring CTFs in this manner represented a much higher
throughput technique for the determination of tractions with platelets. The contractile
forces of many cells could be measured at once instead of individually, thus saving much
time and effort.
Figure 6.3.1 Cells Assembling ECM on Patterned PAA

Cells were placed onto patterned Fn (red) along with fluorescently labeled Fn (green).

After 12 hrs, the media was replaced and the cells were imaged on the surface of the gel.

Scale bar is 20 µm.
Figure 6.3.2 Biomechanical Imaging Experiment and Results
This figure demonstrates how biomechanical imaging is performed using MTM. (A) A parallel plate indenting device is placed on a moving arm above (B) a cell that is on the patterned surface (green dots). The cell contains fluorescent beads (blue) which can be tracked as (F) the substrate is deformed, (G) causing the substrate to stretch and the beads to move with the deformation of the cell. Brightfield and two fluorescent images – one for the fluorescent pattern and one for the phagocytosed beads – of the cell with embedded beads were taken (C) (D) (E) before the cell was subject to stretch and (H) (I) (J) after the cell was stretched. (K) From this information, the shear modulus and
prestress of the cell was able to be measured through an offline analysis. Image adapted from (Canovic et al., 2013) reprinted with permission.
Figure 6.3.3. Platelet On a Fluorescent Fibrinogen Surface.

(A) Human platelets were activated with thrombin and adhered to the PAA substrate that had been patterned with a fluorescent fibrinogen. (B) Platelets were able to contract on the surface of the PAA gels with a known pattern. (C) Confocal images of the cells and patterned fibrinogen are shown. Image courtesy of Wilbur Lam.
APPENDIX 1: TRACTION FORCE ANALYSIS CODE V1

```matlab
%function [p2 initial_file] = 
analyze_initial_image_4(mcircle_dia,post_dist,pixel_ratio)
%Program is to locate and align dots on a grid to match the undeformed
%image
% close all;
% clear;
% m = [];
% pk = [];
% cnt = [];
% a = [];
% b = [];
% pos = [];

mcircle_dia = 1.5; %maximum circle diameter in um
post_dist = 5; %distance between posts in um
pixel_ratio = 0.1075; %number of um per pixel
pixel_dia = round(mcircle_dia / pixel_ratio); %max diameter of pixels
for circle
pix_post_dist = post_dist/pixel_ratio; %distance between posts in
pixels

%Get the file!
initial_file = uigetfile('*.tif', 'Pick The Dot File', 'F:\03_30_11');
initial_file = 'Sum posts glass.tif';

% read in tif file
initial_image = double(imread(initial_file, 'tif'));
% display the image
colormap(gray);
imagesc(initial_image);
freezeColors

% georgetown macros
% for i = 1;
    bp = bpass(initial_image,5,pixel_dia); %bandpass image to remove
background
    m = max(max(bp))*1; % Percent of the brightest feature estimate
    pk = pkfnd(bp, m, pixel_dia); %find all peak locations
    cnt = cntrd(bp,pk,pixel_dia+2); %accurately locate peak centroid
% end

hold;
plot(cnt(:,1),cnt(:,2),'x');

figure
plot(cnt(:,1),cnt(:,2),'x');
```
% selecting points on plot for the corners
datacursormode on;
fig_handle = gcf;
hold on;
pos = zeros(4,2);
for i=1:4,
    choice = menu('Press Enter After Selecting Point','Enter');
    while choice==0
        choice = menu('Press Enter After Selecting Point','Enter');
    end
    % pause();
dcm_obj = datacursormode(fig_handle);
    info = getCursorInfo(dcm_obj);
pos(i,1) = info.Position(1);
pos(i,2) = info.Position(2);
    plot(pos(i,1),pos(i,2), 'rx');
end

% putting the 4 corners into the program
[x,y]=FourCorners(pos(1,:),pos(2,:),pos(3,:),pos(4,:));

% setting time to 0 and 1 so that the program knows which dots to connect
xy = [x,y];
xy(:,3) = 1;

cnt(:,3) = [];
cnt(:,3) = 0;

t_pos = [cnt;xy];
tracked = track(t_pos, 30);

count = 0;
for i = 1: (length(tracked)-1)
    if tracked(i,4) == tracked(i+1,4)
        count = count+1;
        p1(count,1) = tracked(i,1);
        p1(count,2) = tracked(i,2);
        p2(count,1) = tracked(i+1,1);
        p2(count,2) = tracked(i+1,2);
    end
end

"Error" is if there is no displacement, but one can conceive of it to be the
% magnitudes of the displacements
error = sqrt((p1(:,1)-p2(:,1)).^2+(p1(:,2)-p2(:,2)).^2);

cell_image_file = uigetfile('*.tif','Pick The Dot File','F:\03_30_11'); % plotting image for the cell with arrows
% display the figure
figure;
colormap('gray')
% uncomment ONE of the following at a time to show the forces
% for displaying the dot displacement, uncomment
imagesc(double(imread(initial_file)))
% for displaying the displacement on the brightfield image, uncomment
% imagesc(double(imread(initial_file)))
hold;
% John Iverson's Code
freezeColors

A = p1 - p2; % displacements for the image
thresh2 = 0; % threshold displacement of dots in pixels
under = find(error < thresh2); % locating the points under the threshold to set to 0
for i = 1:length(under),
    A(under(i),1) = 0;
    A(under(i),2) = 0;
end

colormap('jet')
% forces are shown at the final position and scaled, if you put the dot % at % the initial position (p2), then unscale with (x,y,u1,u2,0), you can % show % the actual displacements of the dots
quiver(p1(:,1), p1(:,2), A(:,1), A(:,2));

% Get force/displacement vectors magnitudes
Disp = error*pixel_ratio;

% Force magnitudes from gel mechanical data
% Calculate the spring constant from Maloney et al. "Influence of Finite % Thickness % on cellular adhesion-induced deformation of compliant substrata". % Physical Review E. 2008.
% For larger dots or dots that interfere with each other even though % they % are not being pulled on, different parameters need to be used
nu = 0.445; % Poisson's Ratio
dot_radius = 0.6*10^-6; % meters
Elastic_mod = 3658.19; % pascals
k = pi()*(Elastic_mod*dot_radius)/((1+nu)*(2-nu)); % spring constant of gel N/m
k_nano = k*10^3; % nN/um
Force = Disp*k_nano; % nN

% Vectors
D_Vec = A*pixel_ratio;
F_Vec = D_Vec*k_nano;
%%% Setting up plot axes %%

```matlab
N = 5;
max_F = max(Force); % max total Force and cell number
sep = max_F/N; % separation between ticks - (denominator) - 1

% Hard-coded axis labels
num1 = num2str(0,'%6.2f');
num2 = num2str(sep,'%6.2f');
num3 = num2str(sep*2,'%6.2f');
num4 = num2str(sep*3,'%6.2f');
num5 = num2str(sep*4,'%6.2f');
num6 = num2str(sep*5,'%6.2f');

hcb = colorbar('YTickLabel', {num1,num2,num3,num4,num5,num6});

cax_vals = caxis;

cax_width = (cax_vals(2)-cax_vals(1))/5;

set(hcb,'YTick',
[cax_vals(1),cax_vals(1)+cax_width,cax_vals(1)+2*cax_width,...
cax_vals(1)+3*cax_width,cax_vals(1)+4*cax_width,cax_vals(1)+5*cax_width]);
```
function [Xf Yf] = FourCorners(pos1, pos2, pos3, pos4, distance)
%pos1 = pos(1,:)
%pos2 = pos(2,:)
%pos3 = pos(3,:)
%pos4 = pos(4,:)
%The function FourCorners takes in 4 sets of xy pairs representing the
%4
corners of a grid of dots. The idea is to use these 4 corners to
%calibrate
%the distance between the dots in 2 orthogonal axes.
%distance = distance in pixels between dots

%Find the corners
P = [pos1; pos2; pos3; pos4];

dist = zeros(length(P)-1,1);
i=1;
%finding the distance between each point, the longest distance between
%2
%points will be on a diagonal and used to not knock out the pairing
%between
%the first point and that one when drawing the grid
for i = 2:length(P)
    j = i-1;
    dist(j,1) = i;
    dist(j,2) = sqrt((P(1,1)-P(i,1))^2+(P(1,2)-P(i,2))^2);
end

[~,ma] = max(dist(:,2)); %on diagonal
[~,mi] = min(dist(:,2)); %value don't want to use for next one

%find the longest distance not on the diagonal to get the best average
%along the length
for i = 1:length(dist),
    if dist(i,2) ~= max(dist(:,2)) && dist(i,2) ~= min(dist(:,2));
        conn = dist(i,1);
    end
end

set1 = [pos1;P(conn,:)]; %arrange data into sets for theoretically
%parallel lines
set2 = [P(dist(mi,1),:); P(dist(ma,1),:)];

%get equations for both lines
s1 = polyfit(set1(:,1),set1(:,2),1);
s2 = polyfit(set2(:,1),set2(:,2),1);

%manual input
%ask user number of dots between the points
plot(set1(1,1), set1(1,2),'gx');
plot(set1(2,1), set1(2,2),'gx');
s = sprintf('How many spots are between the green points, %d, %d and %d, %d?

set1(1,1), set1(1,2), set1(2,1), set1(2,2));
num_parallel = input(s);

%automatic input
%num_s = sqrt((set1(1,1)-set1(2,1))^2 + (set1(1,2)-set1(2,2))^2);
%num_p = (num_s)/distance;
%num_parallel = round(num_p);

%calculate the x separation between the points on the parallel lines
total_d_parallel_1x = set1(1,1)-set1(2,1);
sep_parallel_1x = abs(total_d_parallel_1x / (num_parallel+1));

total_d_parallel_2x = set2(1,1)-set2(2,1);
sep_parallel_2x = abs(total_d_parallel_2x / (num_parallel+1));

%Preallocating for speed...
c = 2;
x1 = zeros(num_parallel+c,1);
y1 = zeros(num_parallel+c,1);
x2 = zeros(num_parallel+c,1);
y2 = zeros(num_parallel+c,1);

"draw" grid points along each parallel line to dot first line
for i = 1:num_parallel+c,
    if set1(1,1)<set1(2,1) %start a point from the left and go to the right
        x1(i) = set1(1,1)+(i-1)*sep_parallel_1x;
y1(i) = s1(1)*x1(i)+s1(2);
    elseif set1(1,1)>set1(1,2)
        %go backwards if it's not right
        x1(i) = set1(1,1)-(i-1)*sep_parallel_1x;
y1(i) = s1(1)*x1(i)+s1(2);
    end
end

"draw" grid points along each parallel line to dot second line
for i = 1:num_parallel+c,
    if set2(1,1)<set2(2,1) %start a point from the left and go to the right
        x2(i) = set2(1,1)+(i-1)*sep_parallel_2x;
y2(i) = s2(1)*x2(i)+s2(2);
    elseif set2(1,1)>set2(1,2)
        %go backwards if it's not right
        x2(i) = set2(1,1)-(i-1)*sep_parallel_2x;
y2(i) = s2(1)*x2(i)+s2(2);
    end
end
% manual calculate the number of dots to put between
plot(set1(1,1), set1(1,2), 'cx');
plot(set2(1,1), set2(1,2), 'cx');

s = sprintf('How many spots are between the cyan points %d, %d and
%d, %d? \n',...
    set1(1,1), set1(1,2), set2(1,1), set2(1,2));

num_perp = input(s);

% automatic input
num_perp = round((sqrt((set1(1,1)-set2(1,1))^2 + (set1(1,2)-
    set2(1,2))^2))/distance);

Xf = []; Yf = [];
for i = 1:length(x1)
    s = polyfit([x1(i); x2(i)], [y1(i); y2(i)], 1);
    sep_perp_x = abs((x1(i)-x2(i))/(num_perp+1));
    for k = 1:num_perp+2,
        if x1(i)<x2(i) % start a point from the left and go to the right
            X(k) = x1(i)+(k-1)*sep_perp_x;
            Y(k) = s(1)*X(k)+s(2);
        elseif x1(i)>x2(i)
            % go backwards if it's not right
            X(k) = x1(i)-(k-1)*sep_perp_x;
            Y(k) = s(1)*X(k)+s(2);
        end
    end
end
Xf = [Xf; X']; Yf = [Yf; Y'];
end
% end
APPENDIX 2: TRACTION FORCE ANALYSIS CODE V2

function [Traction,disp,xg,yg] =
calculate_disp_maps_v4(cell_image,filename,diam, elast_mod, roi,f,img);

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
% Input: cell_image : An image of the ecm dot pattern
% filename (soon to be obsolete) : This is only used to title
% the figure on which the tractions are plotted.
% roi or A 2 column vector
% [xi(:,),yi(:,)] which defines
% the polygon of interest
% -- from DIC image. If [] is
% supplied, then the entire image is used.
% f
% which the traction vectors are
% case a new figure is generated.
% This is for providing a DIC image on
% will be plotted.
% diam :diameter of the micropatterned dot in um
% stiffness :Young's Modulus of the hydrogel in Pa
% roi : region of interest, if none, can
% use blank []
% img : bright field image or image to be plotted over
%PIXEL SIZE NEEDS TO BE PUT IN AS WELL PLEASE GO INTO THE PROGRAM TO ALTER THE PIXEL

% Output:
%
% Traction : Traction map in nano Newtons(2 column format
% column 1 corresponds to x and column 2 corresponds to y component
% disp : Displacement in microns(2 column format column
% 1 corresponds to x and column 2 corresponds to y component)
% xg,yg : positions at which tractions are calculated

% If you find any bugs please contact:
% Samuel Polio
% Department of Biomedical Engineering,
% Boston University,
% 44 Cummington street,
% Boston, MA 02215. Email : srp215@bu.edu
global theta1 theta2
mcircle_dia = 2;
pixel_ratio = .1075; %60x um/pixel
%pixel_ratio = 0.1613; %40x
pixel_dia = round(mcircle_dia / pixel_ratio); %max diameter of pixels
for circle
    bp = bpass(cell_image,5,pixel_dia); %bandpass image to remove background
    [theta1,theta2, spacing] = Calculate_grid_from_image(bp);
    [xg,yg, spacing2] = estimate_grid_locations(bp);  % <== first estimate of positions
    if(theta1>45) theta1 =theta1-90;end;%theta1 should be > 90 since it would give the same theta otherwise

% ===find actual dot position as intensity weighted centroid of dot
% also fixes for multiple maxima per dot
idx =sub2ind(size(bp),yg,xg); %gives indexes relating to xg and yg to bp
G =zeros(size(bp));G(idx) =1;%puts 1s wherever there are estimates of points
se1 = strel('disk',10); %makes a disk basically with Os and 1s with a radius of 10 pix
L =bwlabel(imdilate(G,se1),8); %takes the image and dialates each pixel marking a location to a white circle
    %labels images larger than 8 units together
G2 =zeros(size(bp)); G2(L>0)=1; G2 =G2.*norm01(cell_image);
%take the bw regions in L and multiplies the original image to find the %regions within a certain area around where the centers were located to %later find the centroid
s = regionprops(L, 'PixelIdxList', 'PixelList');
for loop =1:numel(s);
    idx = s(loop).PixelIdxList;
    pixel_values = double(G2(idx));
    sum_pixel_values = sum(pixel_values);
    x = s(loop).PixelList(:, 1);
    y = s(loop).PixelList(:, 2);
    xbar(loop) = sum(x .* pixel_values) / sum_pixel_values;
    ybar(loop) = sum(y .* pixel_values) / sum_pixel_values;
end;
clear xg yg; xg =xbar; yg =ybar; clear xbar ybar;
% gets new estimates of the centroid from the region of the dots

%-- remove points too close to the border
%-- THRESH is distance in pixels from the border

THRESH =10; % <<--threshold distance in pixels from border
idx1 =find((xg<THRESH));
idx2 =find(xg>size(bp,2)-THRESH);
idx3 =find((yg<THRESH));
idx4 =find(yg>size(bp,1)-THRESH);
idx =setdiff([1:length(xg(:))],union(union(idx1,idx2),union(idx3,idx4)));
% finds all the indexes for xg and yg that are not in the thresholded region
% by looking at the sets of idx1,2,3,4
xg =xg(idx); yg =yg(idx);

clear X2 L G L2 L_with_multiples L_singles X sel ;
%----------------- - -----------------
%===================================
%-----------------------------------

warning off;
if(f==0)
    f =figure;
end;
%figure(f); clf; imshow(norm01(img)); hold on; title(filename);
%show normalized image of figure 1, scaled basically so you can see it

warning('off');
hold on;
%plot positions of final dots
%plot(xg,yg,'b*');
zoom on;
%plot the new centroids on top of the image

%-- grid x
DISTANCE_THRESHOLD_FOR_LINE_FIT = 0.3*spacing;
X_init =[xg(:),yg(:)];
horiz_line_idx =zeros(length(xg),4);
cntr =1;cntr2 =1;
% start at a random point
min_number_pts_for_line_fit =(size(bp,2)/spacing)*0.2;

while(size(X_init,1)>0)
    rand_idx =1;% randi(size(X_init,1));
    x_seed =X_init(rand_idx,1); y_seed =X_init(rand_idx,2); %starts at first x,y coords
    m =tand(-theta1); %theta is from earlier estimate
    c1 =y_seed -m*x_seed; %calculate intercept from given points
    D =abs(X_init(:,2) - X_init(:,1)*m -c1)/sqrt(m*m+1);

    ...
% get estimate of how accurate it is from ests of line parameters m, c
idx = find(D < DISTANCE_THRESHOLD_FOR_LINE_FIT);
% find ideal point with a distance less than the threshold set
% earlier
x_ideal = X_init(idx,1); y_ideal = X_init(idx,2);
% gives array of points that could fit in x and y ideal
[dummy, sort_idx] = sort(x_ideal);
x_ideal = x_ideal(sort_idx); y_ideal = y_ideal(sort_idx);
% organize x and y ideal based on size could definitely be an issue
% for
% large deformations here where it tries to fit points that are
% pulled
% into the region, but may not be actually associated with that line

% ----- repeat this
clf = mean(y_ideal - m*x_ideal);
D = abs(X_init(:,2) - X_init(:,1)*m - clf)/sqrt(m*m + 1);
% tries to get a better bit based on all the points that were within
% the
% tolerance of D
% not sure if i like the +1... maybe should be another condition to
% fix
% this since 1 may dominate in some cases? but then again mostly
% won't
% matter since if it was close to 0, want to divide by 1 anyways
idx = find(D < DISTANCE_THRESHOLD_FOR_LINE_FIT);

if(length(x_ideal) > 5)
tot_lines{cntr2} = [x_ideal, y_ideal];
cntr2 = cntr2 + 1;
end;

if(length(x_ideal) > min_number_pts_for_line_fit)
% no previous reference??? so it will always do this basically
lines{cntr} = [x_ideal, y_ideal];
[m, c1, R_sq, SE_slope, SE_ip] = fit_line(x_ideal, y_ideal);
clf = mean(y_ideal - m*x_ideal);
new_idx = find(ismember([xg, yg], [x_ideal, y_ideal], 'rows'));
horiz_line_idx(new_idx, 1) = cntr;
horiz_line_idx(new_idx, 2) = c1;
horiz_line_idx(new_idx, 3) = m;
horiz_line_idx(new_idx, 4) = abs(y_ideal - x_ideal*m -
c1)/sqrt(m*m + 1);
cntr = cntr + 1;

% y_ideal = m*x_ideal + c1; -- only for plot;
end;

X_init = setdiff(X_init, X_init(idx, :), 'rows');
% returns the rows in X_init that are not part of X_init(idx, :)
end;

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
GRID FITTING for X %%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%------------------------------------------------%
% 1) sort the lines from min y intercept to max y intercept
ips = unique(horiz_line_idx(:, 2));
% finds all the unique intercepts for the lines
ips = sort(ips(ips > 0));
% sort all intercepts that are nonzero
ipsd = diff(ips); % [mean(ipsd), std(ipsd) std(ipsd)/mean(ipsd) *100]
ips = sort(ips);
% ipsd sorting? already have ips sorted from previous changes
lines2 = cell(length(lines), 1);
for i = 1:length(ips)
    idx = unique(horiz_line_idx(find(horiz_line_idx(:, 2) == ips(i)), 1));
    % find the unique line index corresponding to the intercept,
    % basically
    % the center to start at
    lines2{i} = lines{idx};
    % organizes the lines themselves into lines2 in order of the
    intercept
    slope_h(i) = unique(horiz_line_idx(find(horiz_line_idx(:, 2) == ips(i)), 3));
    % grabs all the slopes matching the ips
    horiz_line_idx(find(horiz_line_idx(:, 2) == ips(i)), 1)
    = horiz_line_idx(find(horiz_line_idx(:, 2) == ips(i)), 1)/unique(horiz_line_idx
    (find(horiz_line_idx(:, 2) == ips(i)), 1));
    horiz_line_idx(find(horiz_line_idx(:, 2) == ips(i)), 1) = i;
    % redefines it to be a scalar? something is wrong here. should
probably
    % have the previous line commented out
    err_h(i) = sum(horiz_line_idx(find(horiz_line_idx(:, 2) == ips(i)), 4));
    % column 4 is the diff between the points and the estimate of the
grid
end;
slope_h = mean(slope_h) * (slope_h ./ slope_h);
%slope_h ./ slope_h gives 1? I guess gives same length of vector for mean
%slope of the lines
lines = lines2; clear lines2;
h_spacing = mean(ipsd);

global lines
[IP_M_SPACING, fval, exitflag] = fminsearch(@grid_fitting_function, [ips(1), unique(slope_h), h_spacing]);
if(exitflag == 1)
    sprintf('%s', 'Horizontal spacing minimized ok!')
elseif(exitflag == 0)
    sprintf('%s', 'Horizontal spacing minimization MAX ITERATIONS EXCEEDED')
elseif(exitflag == -1)
    sprintf('%s', 'Horizontal spacing minimization... Algorithm terminated by the output function')
end;

h_spacing = IP_M_SPACING(3);
sze(h_spacing)
slope_h = IP_M_SPACING(2);
sze(slope_h)
ip_ideal = IP_M_SPACING(1) + [0:length(lines)] * h_spacing;
size(ip_ideal)

% for i = 1:length(ip_ideal)
%    dummy = lines{i};
%    x_ideal = dummy(:, 1);
%    err_h(i) = sum(abs(y_ideal - x_ideal * slope_h(i) -
%        ip_ideal(i)) / sqrt(slope_h(i) * slope_h(i) + 1));
%    %hold on; plot(x_ideal, slope_h * x_ideal + ip_ideal(i), 'y-');
%    %pause(0.5);
%    bef = ceil(min(x_ideal) / abs(h_spacing)); % number of points before
%    % the start
%    aft = ceil((size(img, 2) - max(x_ideal)) / abs(h_spacing)); % numb
%    points after end
%    a_ideal = [1:aft]' * abs(h_spacing) + max(x_ideal);
%    x_ideal2 = [b_ideal; x_ideal; a_ideal];
%    hold on; plot(x_ideal2, slope_h * x_ideal2 + ip_ideal(i), 'b-');
% end;

for i = 1:size(horiz_line_idx, 1);
    idx = horiz_line_idx(i, 1);
    if(idx == 0) continue; end;
    horiz_line_idx(i, 2) = ip_ideal(idx);
    horiz_line_idx(i, 3) = slope_h;
end;

clear ipy_ideal xl
ipy_ideal2(:, 1) = IP_M_SPACING(1) + [-5:length(lines) + 5] * h_spacing;
ipy_ideal2(:, 2) = ones(length(ipy_ideal2(:, 1)), 1) * slope_h;
xl = 1:1:size(img, 2);

%plot new gridlines
%for i = 1:length(ipy_ideal2(:, 1)),
%    hold on; plot(xl, slope_h * xl + ipy_ideal2(i, 1), 'r-');
theta1 = theta1 + 90;
min_number_pts_for_line_fit = 3;

% -- grid x
X_init = [xg(:), yg(:)];
cntr = 1; cntr2 = 1;
% start at a random point
vert_line_idx = zeros(length(xg), 4);
while (size(X_init, 1) > 0)
    rand_idx = 1; % randi(size(X_init, 1));
    x_seed = X_init(rand_idx, 1);
    y_seed = X_init(rand_idx, 2);
    if (theta1 < 90)
        m = tand(-theta1);
        c1 = y_seed - m * x_seed;
    end
    D = abs(X_init(:, 2) - X_init(:, 1) * m - c1) / sqrt(m * m + 1);
    idx = find(D < DISTANCE_THRESHOLD_FOR_LINE_FIT);
    x_ideal = X_init(idx, 1);
    y_ideal = X_init(idx, 2);
    [dummy, sort_idx] = sort(x_ideal);
    x_ideal = x_ideal(sort_idx);
    y_ideal = y_ideal(sort_idx);
    c1 = mean(y_ideal - m * x_ideal);
    D = abs(X_init(:, 2) - X_init(:, 1) * m - c1) / sqrt(m * m + 1);
    idx = find(D < DISTANCE_THRESHOLD_FOR_LINE_FIT);
    x_ideal = X_init(idx, 1);
    y_ideal = X_init(idx, 2);
    [dummy, sort_idx] = sort(x_ideal);
    x_ideal = x_ideal(sort_idx);
    y_ideal = y_ideal(sort_idx);

    if (length(x_ideal) >= 5)
        tot_lines{cntr2} = [x_ideal, y_ideal];
        cntr2 = cntr2 + 1;
    end

    if (length(x_ideal) >= min_number_pts_for_line_fit) % No
        linesv{cntr} = [x_ideal, y_ideal];
        [m, c1, R_sq, SE_slope, SE_ip] = fit_line(x_ideal, y_ideal);
        c1 = mean(y_ideal - m * x_ideal);
        new_idx = find(ismember([xg, yg, x_ideal, y_ideal, 'rows']));
        vert_line_idx(new_idx, 1) = cntr;
        vert_line_idx(new_idx, 2) = c1;
        vert_line_idx(new_idx, 3) = m;
        vert_line_idx(new_idx, 4) = abs(y_ideal - x_ideal * m - c1) / sqrt(m * m + 1);
    end
cntr =cntr+1;
end;

X_init =setdiff(X_init,X_init(idx,:),'rows');
end;

%%%%%%%%%%%%%%%%%%%%%%%%% GRID FITTING Y %%%%%%%%%%%%%%%%%%%%%
%------------------------------------------------% 
% 1) sort the lines from min y intercept to max y intercept 
ips =unique(vert_line_idx(:,2)); ips =sort(ips(ips==0)); ipsd =diff(ips); %[mean(ipsd), std(ipsd) std(ipsd)/mean(ipsd)*100] 
ips=sort(ips, 'descend');
linesv2 =cell(length(linesv),1);
for i =1:length(ips)
    idx =unique(vert_line_idx(find(vert_line_idx(:,2)==ips(i)),1));
    linesv2(i) = linesv(idx);
    slope_v(i)=
    unique(vert_line_idx(find(vert_line_idx(:,2)==ips(i)),3));
    vert_line_idx(find(vert_line_idx(:,2)==ips(i)),1)
    =vert_line_idx(find(vert_line_idx(:,2)==ips(i)),1)/unique(vert_line_idx
    (find(vert_line_idx(:,2)==ips(i)),1));
    err_v(i)= sum(vert_line_idx(find(vert_line_idx(:,2)==ips(i)),4));
end;

slope_v =mean(slope_v) *(slope_v./slope_v);
linesv =linesv2; clear linesv2;
v_spacing =mean(ipsd);

global linesv
[IP_M_SPACING,fval,exitflag] = 
fminsearch(@grid_fitting_function_vert,[ips(1),unique(slope_v),v_spacing]);

if(exitflag ==1)
    sprintf('%s', 'Vertical spacing minimized ok!')
elseif(exitflag ==0)
    sprintf('%s', 'Vertical spacing minimization MAX ITERATIONS EXCEEDED')
elseif(exitflag ==-1)
    sprintf('%s', 'Vertical spacing minimization... Algorithm terminated by the output function')
end;

v_spacing=IP_M_SPACING(3);
slope_v=IP_M_SPACING(2);
% this needs to be added to in order to make more lines
ip_ideal = IP_M_SPACING(1) + [0:length(linesv)] * v_spacing;

% for i = 1:length(ip_ideal)
  dummy = linesv{i};
  x_ideal = dummy(:,1);
  % err_h(i) = sum(abs(y_ideal - x_ideal * slope_h(i) -
                    ip_ideal(i)) / sqrt(slope_h(i) * slope_h(i) + 1));
  % hold on; plot(x_ideal, slope_v * x_ideal + ip_ideal(i), 'r-
  pause(0.5);
  % lengthen the line in order to make sure it covers the entire
  image
  bef = ceil(min(x_ideal) / abs(v_spacing)); % number of points before
  the start
  b_ideal = [1:1:bef]' * abs(v_spacing) + min(x_ideal);
  aft = ceil((size(img,2) - max(x_ideal)) / abs(v_spacing)); % numb
  % points after end
  a_ideal = [1:1:aft]' * abs(v_spacing) + max(x_ideal);
  x_ideal2 = [b_ideal; x_ideal; a_ideal]; % makes the longer line over
  the whole image / area
  % hold on; plot(x_ideal2, slope_v * x_ideal2 + ip_ideal(i), 'r-
  end;

for i = 1:size(vert_line_idx,1);
  idx = vert_line_idx(i,1);
  if(idx==0) continue;end;
  vert_line_idx(i,2) = ip_ideal(idx);
  vert_line_idx(i,3) = slope_v;
end;

% trying to make spacing for more lines than given in program
ipx_ideal2(:,1) = IP_M_SPACING(1) + [-5:length(linesv)+5] * v_spacing;
ipx_ideal2(:,2) = ones(length(ipx_ideal2(:,1)),1) * slope_v;
x1 = 1:1:size(img,2);
% for i = 1:length(ipx_ideal2(:,1)),
  % hold on; plot(x1, slope_v * x1 + ipx_ideal2(i,1), 'r-
% end

% finding the intercepts of all the lines to make the extended grid
count = 1;
for i = 1:length(ipx_ideal2(:,1))
  cl = ipx_ideal2(i,1); ml = slope_v;
  for j = 1:length(ipy_ideal2(:,1))
    c2 = ipy_ideal2(j,1); m2 = slope_h;
    P(count,1) = (c2 - cl) / (m1 - m2);
    P(count,2) = (m2 * c1 - m1 * c2) / (m2 - m1);
    count = count + 1;
  end
end
% hold on; plot(P(:,1), P(:,2), 'r*');
% commented out section because won't work with old tracking algorithm for larger dot displacements
P = ones(length(xg), 2) * nan;
for i = 1:length(xg)
  if((horiz_line_idx(i, 1) == 0) || (vert_line_idx(i, 1) == 0))
    continue;
  end;
  c1 = horiz_line_idx(i, 2); c2 = vert_line_idx(i, 2);
  m1 = horiz_line_idx(i, 3); m2 = vert_line_idx(i, 3);
  P(i, 1) = (c2 - c1) / (m1 - m2);
  P(i, 2) = (m1 * c2 - m2 * c1) / (m1 - m2);
end

% making a large square that will have n*m points of intersection
for i = 1:(length(horiz_line_idx) * length(vert_line_idx))

% setting up tracking
P(:, 3) = 0;
X = [xg(:, 1), yg(:, 1), ones(length(xg), 1)];
hold on; plot(P(:, 1), P(:, 2), 'r*');
disp = [X - P] * pixel_ratio;

% put all into one vector to connect dots
tr = [P; X];
tracked = track(tr, 28);

% connect tracks
count = 0;
for i = 1:(length(tracked) - 1)
  if tracked(i, 4) == tracked(i + 1, 4)
    count = count + 1;
    p1(count, 1) = tracked(i, 1);
    p1(count, 2) = tracked(i, 2);
    p2(count, 1) = tracked(i + 1, 1);
    p2(count, 2) = tracked(i + 1, 2);
  end
end

pixel_ratio = 0.1613;
disp = [p2 - p1] * pixel_ratio;
m = (sqrt(disp(:, 1).^2 + disp(:, 2).^2));
P = p1;
X = p2;

%plot initial positions
hold on; %plot(P(:,1),P(:,2),'*');

% -- Traction calculation
% -- From Sam Polio
%-----------------------------
pixel_dia = round(mcircle_dia / pixel_ratio); %max diameter of pixels for circle
%Get force/displacement error vectors in um
%Force magnitudes from gel mechanical data
nu = 0.445; %poisson's ratio
dot_radius = diam/2*10^-6; %m
%elast_mod = 3658.19; %0.07% Pa
%elast_mod = 7600; %0.13% Pa

k = pi()*(elast_mod*dot_radius)/((1+nu)*(2-nu)); %N/m
k_nano = k*10^-3; %nN/um

size(k_nano)
size(disp)
Traction = disp*k_nano;%nN

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
m = ( (sqrt (Traction(:,1).^2+Traction(:,2).^2)));
disp2 = disp;
disp2(:,1) = disp2(:,1)/max(m); disp2(:,2) = disp2(:,2)/max(m);

disp_x = disp2(:,1)*sp.*0.75;
disp_y = disp2(:,2)*sp.*0.75;

% freezeColors
% colormap('jet')
% quivc(P(:,1),P(:,2),disp(:,1),disp(:,2));
% if(isempty(roi))
% in =1:length(m);
% else
% xi = roi(:,1); yi = roi(:,2);
% hold on; %plot(xi,yi,'g','LineWidth',2);
% in = inpolygon(P(:,1),P(:,2),xi,yi);
% end;

% hold on; %plot(P(in,1),P(in,2),'*'); P(-in,1),P(-in,2),'*')
% %set(gca,'CLim',([min(m(in)),max(m(in)))]);
% %set(gca,'CLim',([0,max(sqrt(Traction(:,1).^2+Traction(:,2).^2))]));
% colorbar;
% 
% figure;
% colormap('gray')
% imagesc(img)
% title('Force Vectors')

hold on;
freezeColors;
colormap('jet');

% want to add a scaling vector in upper right corner
P = [P; 20, 50];  % add position of vector in pixels
FM = 10;  % scale bar length
L = FM/k_nano;  % length of vector

disp = [disp; L, 0];  % length of vector in um

% Filtering out displacements less than set amount (pix)
% pix = 0.3;
% for i = 1:length(disp),
% if sqrt(disp(i,1).^2+disp(i,2).^2) < pix;
% disp(i,1) = 0;
% disp(i,2) = 0;
% end
% end

% plot the displacements in color
quiverc(P(:,1),P(:,2),disp(:,1),disp(:,2));
axis off;

% Set the limits of the colorbar to match the force magnitudes
N = 5;
max_F = max(sqrt(disp(:,1).^2+disp(:,2).^2)*k_nano);  % max total Force and cell number

sep = max_F/N;  % separation between ticks (denominator) - 1

num1 = num2str(0,'%6.2f');
num2 = num2str(sep,'%6.2f');
num3 = num2str(sep*2,'%6.2f');
num4 = num2str(sep*3,'%6.2f');
num5 = num2str(sep*4,'%6.2f');
num6 = num2str(sep*5,'%6.2f');

hcb2 = colorbar('YTickLabel', {num1,num2,num3,num4,num5,num6});
cax_vals = caxis;
cax_width = (cax_vals(2)-cax_vals(1))/5;
set(hcb2,'YTick',
[cax_vals(1),cax_vals(1)+cax_width,cax_vals(1)+2*cax_width,...
cax_vals(1)+3*cax_width,cax_vals(1)+4*cax_width,cax_vals(1)+5*cax_width]);
set(gca,'YDir','reverse');
clear global;
function [theta1, theta2, spacing] = Calculate_grid_from_image(phantom)
% Calculate_grid_from_image gives the dot spacing and angles from the image itself without any fitting.
% Phantom: Image of Fluorescent dots to be analyzed, should be bp
% phantom is the bp image and theta is the angle, but theta is defined in the program so it seems to be superfluous, so I removed it (phantom, theta)
% theta1 and theta2 are the same when returned, so maybe consider removing % in later version
% spacing is the spacing between the peaks that occurs most frequently in
% the radon transform image

% Y = round( (norm01(cnt(:,1)) * (imgsz(2) - 1)) +1);
% X = round( (norm01(cnt(:,2)) * (imgsz(1) - 1)) +1);
%
% phantom = zeros(imgsz);
% ind = sub2ind(img_sz, X, Y);
% phantom(ind) = 1;
% se = strel('disk',3);
% phantom2 = imdilate(phantom, se);
% theta = 0:1:180;
% [R, xp] = radon(phantom2, theta);
% d = bwdist(phantom); grid_spacing = mode(d)

theta = 0:1:180; % defines theta for radon transform
[R, xp] = radon(phantom, theta);
% radon computes the radon transform of the image, basically it linescans the image at different angles to obtain the line directions / peaks

figure;
imshow(R, [], 'Xdata', theta, 'Ydata', xp, ...'
' InitialMagnification', 'fit')
xlabel('	heta (degrees)')
ylabel('x')
colormap(hot), colorbar; axis square;
iptsetpref('ImshowAxesVisible', 'off'); axis on

% R's columns contains the value radon transform for each angle in theta % the center pixel of the image is the image size / 2 to give x' = 0 % so basically there's 3D data - x is theta, y is r', z is intensity of radon transform at a distance x
R2=max(R,[],1); % [Rs, idx] = sort(R2, 'descend'); theta2 = theta(idx(1:2));
% gets maximum from each column of the transform, so basically this
would be % a maximum at each theta

[maxtab, -, -]=peakdet(R2, mean(R2)+2*std(R2));
% peakdet determines the local max and mins using the column maxes
% mean+sd is the min difference between the peak and surrounding to be
% declared a peak - in this case, the peaks will correspond to the two
% thetas that should be 90 deg apart, don't worry about max_min
% col 1 of maxtab is the index of R2 and col 2 is the value

[-, idx] = max(maxtab(:,2));
% if more than one max determined, find the maximum value of the peaks
theta1 = theta(maxtab(idx,1));
% so the index of the max value gives the angle of the dots
theta2 = theta1;
% same as theta1? assume that this has something to do with the
% commented
% stuff below

idx1 = maxtab(idx,1);
% index of theta that's being looked at for the strongest peak

% theta2 = setdiff( theta(maxtab(:,1)), theta1 );
% [mn, idx] = min(abs((theta2-theta1) -90));
% theta2 = theta2(idx);

R1=R(:,idx1);
% column corresponding to the index

[maxtab, -, -]= peakdet(R1, mean(R1)+std(R1));
% find peaks corresponding to the distance between each row

spacing = mode( diff(maxtab(:,1)) )
% the spacing is the most frequently occurring difference between the
% consecutive points
function [xg, yg, spacing2] = estimate_grid_locations(bp); % offsetx, offsety;
% estimate_grid_locations will find the initial positions of the points
% in
% the grid and give a second estimate of the spacing
% ctimshow(1-norm1(bp)); hold on;
% figure;
% colormap(gray);
% imagesc(initial_image);
% freezeColors

[maxtab, -, -] = peakdet(bp(:, mean(bp(:)) + 2*std(bp(:)));
% find the peaks of the bandpassed image

[j1, i1] = ind2sub(size(bp), maxtab(:, 1));
% gives row and column numbers according to what bp is for a given index
% p = plot(i1, j1, 'r*'); hold on;

bp = bp';
% transpose of bp
[maxtab, -, -] = peakdet(bp(:, mean(bp(:)) + 2*std(bp(:)));
[i2, j2] = ind2sub(size(bp), maxtab(:, 1));

% p = plot(i2, j2, 'g*');
T = intersect([i1, j1], [i2, j2], 'rows');
spacing2 = mode(diff(maxtab));
xg = T(:, 1); yg = T(:, 2);
% p = plot(i, j, 'g*');
bp = bp';
function [slope, ip, R_sq, SE_slope, SE_ip] = fit_line(x_axis, y_axis)
% [slope, ip, R_sq, SE_slope, SE_ip] = fit_line(x_axis, y_axis)
x_axis = x_axis(:);
y_axis = y_axis(:);

p = polyfit(x_axis, y_axis, 1);
slope = p(1);
ip = p(2);

Sxx = sum((x_axis - mean(x_axis)).^2);
Syy = sum((y_axis - mean(y_axis)).^2);
Sxy = sum((y_axis - mean(y_axis)).*(x_axis - mean(x_axis)));
R_sq = Sxy^2/(Sxx*Syy);

s = sqrt((Syy - (Sxy^2)/Sxx)/(length(x_axis)-2));
SE_slope = s/sqrt(Sxx);
SE_ip = s*(sqrt((1/length(x_axis)) + ((mean(x_axis)^2)/Sxx)));
function err = grid_fitting_function(IP_M_SPACING)

global lines;
ip0 = IP_M_SPACING(1);
slope_h = IP_M_SPACING(2);
h_spacing = IP_M_SPACING(3);

ipIdeal = ip0 + [0:length(lines)-1]*h_spacing;

for i = 1:length(ipIdeal)
    dummy = lines{i};
    y_ideal = dummy(:, 2); x_ideal = dummy(:, 1);
    err_h(i) = sum(abs(y_ideal - x_ideal*slope_h -
                    ipIdeal(i)))/sqrt(slope_h*slope_h+1));
    % hold on; plot(x_ideal, slope_h(i)*x_ideal+ipIdeal(i), 'ms-');
    pause(0.5);
end;
err = sum(err_h(:));
function err = grid_fitting_function_vert(IP_M_SPACING)

global linesv;
ip0 = IP_M_SPACING(1);
slope_h = IP_M_SPACING(2);
h_spacing = IP_M_SPACING(3);

ip_ideal = ip0 + [0:length(linesv)-1]*h_spacing;

for i = 1:length(ip_ideal)
dummy = linesv{1};
y_ideal = dummy(:,2); x_ideal = dummy(:,1);
err_h(i) = sum(abs(y_ideal - x_ideal*slope_h-ip_ideal(i)))/sqrt(slope_h*slope_h+1));
% hold on; plot(x_ideal,slope_h(i)*x_ideal+ip_ideal(i),'ms-');
pause(0.5);
end;
err = sum(err_h(:));
function vectn=norm01(vect)

%norm01 takes the input and normalizes the image

vect =double(vect);

mn =min(vect(:));%finds maximum of a large column basically
mx =max(vect(:));
if((mx==0)&(mx==mn)) %normalizes based on contingencies of max = min
    vectn = vect/mx;
elseif(mx==mn)
    vectn = vect;
else
    vectn = (vect -mn)./(mx-mn);
end;
function [maxtab, mintab, max_min] = peakdet(v, delta, x)
% PEAKDET Detect peaks in a vector
% [MAXTAB, MINTAB] = PEAKDET(V, DELTA) finds the local
% maxima and minima ("peaks") in the vector V.
% MAXTAB and MINTAB consists of two columns. Column 1
% contains indices in V, and column 2 the found values.
% With [MAXTAB, MINTAB] = PEAKDET(V, DELTA, X) the indices
% in MAXTAB and MINTAB are replaced with the corresponding
% X-values.
% A point is considered a maximum peak if it has the maximal
% value, and was preceded (to the left) by a value lower by
% DELTA.

% Eli Billauer, 3.4.05 (Explicitly not copyrighted).
% This function is released to the public domain; Any use is allowed.

maxtab = []; mintab = [];

v = v(:); % Just in case this wasn't a proper vector

if nargin < 3
    x = (1:length(v))';
else
    x = x(:);
    if length(v) ~= length(x)
        error('Input vectors v and x must have same length');
    end
end

if (length(delta(:))) > 1
    error('Input argument DELTA must be a scalar');
end

if delta <= 0
    error('Input argument DELTA must be positive');
end

mn = Inf; mx = -Inf;
mnpos = NaN; mxpos = NaN;

lookformax = 1;
max_min = [];
for i = 1:length(v)
    this = v(i);
    if this > mx, mx = this; mxpos = x(i); end
    if this < mn, mn = this; mnpos = x(i); end

    if lookformax
if this < mx-delta
    maxtab = [maxtab ; mxpos mx];
    max_min = [max_min; mxpos mx];
    mn = this; mnpos = x(i);
    lookformax = 0;
end
else
    if this > mn+delta
        mintab = [mintab ; mnpos mn];
        max_min = [max_min; mnpos mn];
        mx = this; mxpos = x(i);
        lookformax = 1;
    end
end
end
BIBLIOGRAPHY


subcellular resolution. Biomechanics and Modeling Mechanobiology [Epub ahead of print]


CURRICULUM VITAE

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Objective
I am seeking a postdoctoral position to obtain experience so that I may pursue a future career in academia. My current research has greatly developed my experimental skills and problem solving abilities, greatly developing me as a scientist. I hope to be able to apply my skills to challenging problems and continue this process on my way to becoming a fully independent investigator.

EDUCATION
Boston University, Boston, MA
College of Engineering, Department of Biomedical Engineering
MS/PhD Program
May 2014

Boston University, Boston, MA
College of Engineering, Department of Biomedical Engineering
Bachelor of Science, Biomedical Engineering, Summa Cum Laude
2009

RESEARCH EXPERIENCE
Graduate Research Student
Matrix Mechanotransduction Lab at Boston University
Boston, MA
May 2010 to Present

• Developing a novel system for the measurement of cellular traction forces for the probing
  of cellular interactions with their extracellular environment.
• Using soft lithography techniques to create microfabricated stamps for microcontact
  printing fluorescently labeled proteins.

BU Senior Project and Internship
TIME Lab, Brigham and Women's Hospital
Boston, MA

• Senior project work on 3D freeform fabrication techniques.

Research and Development Intern
TEI Biosciences Inc.
Boston, MA
June 2008 to August 2008

• Exploration and mechanical testing of improvements to existing products derived from
  bovine dermis for novel applications for ligament repair.

Research Intern
Core Laboratory, Boston Children’s Hospital
Boston, MA
May 2004 to June 2008

• Researching and developing a clinical assay for 25-dihydroxyvitamin D using column
  chromatography and liquid chromatography, tandem mass spectrometry (LC-MS/MS).
AWARDS AND HONORS
BMES Travel Award 2012
Engineering Scholar Scholarship 2005-2009

PUBLICATIONS

PUBLISHED PROCEEDINGS AND ABSTRACTS

**TEACHING EXPERIENCE**

Lab Teaching Assistant. ENG BE 491. Engineering Physiology. Fall 2010

**MENTORSHIP EXPERIENCE**

- Co-mentored, with Dr. Smith, multiple Biomedical Engineering Senior Design teams
- Hands on technical dissemination of microcontact printing and traction force microscopy protocols and other adaptions to multiple collaborating labs (Bermudez Lab, UMass Amherst; Lam Lab, Georgia Tech; Sharma Lab, University of Bath)
- Guiding rotating and new graduate and undergraduate students within the Matrix Mechanotransduction Lab

**ORGANIZATIONS AND ACTIVITIES**

Biomedical Engineering Society Student Member 2012-Present
NIH QBP Student Representative 2012 – 2013
Graduate Assistance in Areas of National Need Fellowship in Nanotechnology 2011-2012
National Institute of Health Training Program in Quantitative Biology and Physiology 2009-Present