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Real-time Analysis of Cell Traction Forces *In vitro* on Deformable Hydrogels

A Major Qualifying Project Report

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By

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Authorship

This report was the result of a group effort by our entire team, and represents our combined efforts and writing. Although one person was the primary author of each section, these sections were reviewed thoroughly by the whole group

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Abstract

Cell traction forces (CTFs) are important to many biological processes such as tissue homeostasis, cell migration and tumor metastasis. Cell traction force microscopy (CTFM) is a well-established method to analyze CTF by measuring the CTF-induced deformation of elastic hydrogels embedded with fluorescent beads on the surface. The measurement is accomplished by imaging the bead configuration with and without CTFs and tracing the displacements of fluorescent beads. However, current CTFM requires cells to be removed from the substrate, which prohibits real-time monitoring of CTFs during culture. Based on CTFM, we have developed a method to measure CTFs in real time over extended periods, permitting reincubation between multiple measurements. Our method relies upon a location indicator system composed of a micro-patterned grid with reference fluorescence beads. The system allows for imaging the bead configuration on a large area of the gel surface before seeding cells. The corresponding unloaded bead configuration beneath the cells can then be acquired, from which the deformation of the gel and CTFs can be calculated. Our preliminary results show that our method succeeded in measuring CTFs of NIH/3T3 fibroblasts multiple times over 24 hours and we can expect various future applications to be explored.

Chapter 1: Introduction

Cell traction forces (CTFs) are important for the understanding of many cellular processes such as cell migration, differentiation, wound healing, and tumor metastasis. Cells migrate on a substrate by attaching to multiple sites through their extracellular matrix and by binding to integrin proteins on a substrate. CTF is applied through an actomyosin mechanism internal to the cell after substrate attachment (Janmey *et al*, 2009). Cell contraction occurs inwardly using the integrin as an anchor point allowing motion (Wang and Lin, 2007). Measuring traction forces allows for important characteristics of cells to be understood in relation to diseases (Janmey *et al*, 2009). Real-time analysis has great potential in testing of pharmaceuticals, substrate stiffness, and related cellular processes of different cell types.

Current methods for cell traction force microscopy (CTFM) involves seeding cells onto fluorescent bead embedded deformable hydrogels, imaging fluorescent bead configurations soon after cell seeding and after the removal of the cells followed by CTF determination using finite element analysis. The limitation of this method is that it involves removing the cell from the substrate which prohibits real-time analysis of CTFs. Our project was aimed at addressing this limitation.

Based on CTFM, we have developed a method to measure CTFs in real time over extended periods, permitting reincubation between multiple measurements. In order to allow CTF measurement in real-time, our approach was to create a location indicator system on hydrogels used for CTFM. To achieve this, imaging of the fluorescent bead configuration on a large area was performed in advance. During the experiment, the location of a selected cell can be precisely identified from the original image. The positional shifts of the fluorescent beads due

to cell contraction can then be determined. The position identification system has two components: a stamped micro-grid with coordinates, and a set of red and green fluorescent beads as location indicators.

Chapter 2: Literature Review

Cell traction forces are important for understanding many cell functions and processes such as cell migration, differentiation, wound healing, and tumor metastasis. Measuring cell traction forces poses a unique problem that can be approached through different methods. One method for measuring cell traction force is through the usage of deformable hydrogels. The link between deformable hydrogels stiffness and cell traction force is also important to the measurements and must be taken into consideration.

Cell traction force plays an important role in different biological processes. Wound healing is greatly affected by cell traction force through the relationship between fibroblast migration and scar tissue formation (Janmey *et al*, 2009). Stiffening of the liver through cirrhosis and fibrosis also may implicate cell traction force as a cause in disease progression. The relationship between tissue stiffness and cell motion sets the stage for research into cell traction forces.

Cells move on a substrate by attaching to multiple sites called focal adhesions. Focal adhesions are where the extracellular matrix binds with integrin on a substrate. Inside the cell actomyosin is connected to different types of tyrosine kinase enzymes. Tyrosine kinase acts as a signaling enzyme for the cell. The tyrosine kinase is then attached to integrin proteins which are linked to the substrate which the cell is on. The integrin proteins play a crucial role in cell

movement by connecting the extracellular matrix of the cell with the substrate and internal system of the cell. The migration of a cell through focal adhesions is illustrated in Figure 2-1(Wang and Lin, 2007).

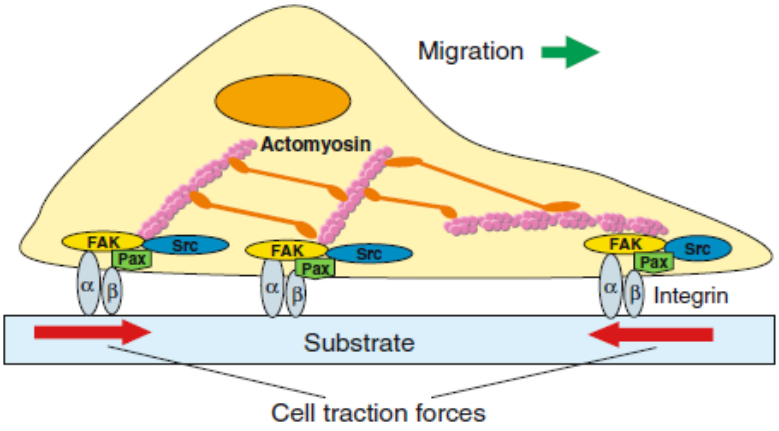


Figure 2-1: Illustration of cell traction force (Wang and Lin, 2007)

Movement of the cell is caused by the actomyosin contraction triggering the tyrosine kinase enzymes. The tyrosine kinase enzymes being the connection between the integrin and cell act as a signal and the cell begins to contract. Cell contraction occurs inwardly using the integrin as an anchor point allowing motion. The motion of cells is also affected by the surrounding environment and its properties.

The environment or the substrate the cell is placed on also has an effect on the motion of a cell and its traction force. The mechanical properties of the substrate, mainly the stiffness, can affect cell migration and can result in different cell shapes (Janmey *et al*, 2009). Substrates which are rigid, such as a plastic dish, result in cells having more focal adhesions which can mean that the cells will be elongated and exert more force when migrating (Wells, 2008).

An *in vitro* model of substrate stiffness can be achieved by varying substrate stiffness which can yield more accurate cell traction force measurements. By using a hydrogel made of polyacrylamide and controlling stiffness through varying the concentrations of a cross linker bis-acrylamide, different cell traction force measurements can be done. The reason for using deformable hydrogels is because plastic and glass substrates have a stiffness that is much stiffer than most tissues in the human body (Wells, 2008).

Currently, a well-established method based deformable hydrogel for measuring cell traction force is called Cell Traction Force Microscopy (CTFM). Fluorescent beads are embedded on the top surface of the hydrogel. Cells are seeded on the gel surface and apply traction force on the gel. By tracking the displacement of the beads, the deformation of the gel can be measured and cell traction forces can be calculated. This method has been used extensively in numerous studies and has yielded satisfactory results (Wang and Lin, 2007). Figure 2-2 is an example of how CTFM is conducted.

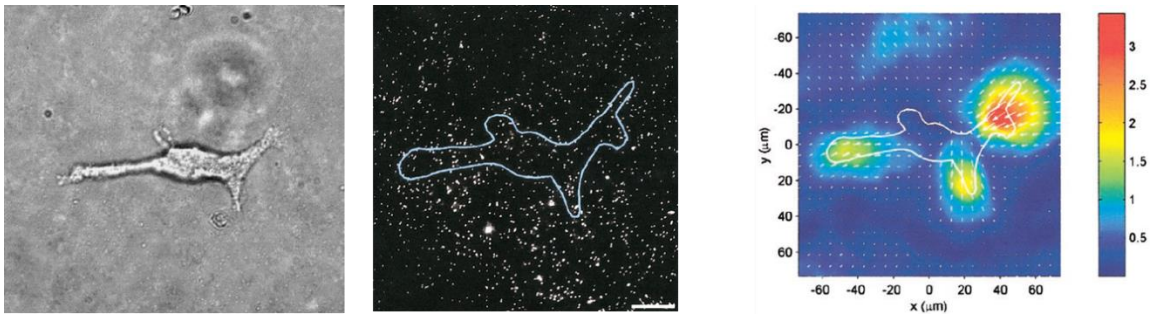


Figure 2-2: Cell Traction Force Microscopy Example (Butler *et al*, 2002)

However, the conventional CTFM requires the cells to be removed from the gel surface in order to provide a comparison between the loaded and relaxed states of the gel. As a result, it allows only single time measurement for a sample. An improvement over the old method that enables multiple measurements of CTF would facilitate researches in various fields.

Besides CTFM, several other methods have been used to measure cell traction force, including: cell populated collagen gel, thin silicone membrane, and force sensor array methods. Each method has different components and some do not use a polyacrylamide gel as our method does. Examining the different methods reveals a need for a new technique.

The first method involves the usage of a cell populated collagen gel. The method requires mixing a collagen gel with cells to form a disk. The disk then contracts when cell traction forces are applied causing the diameter of the disk to change (Bell *et al*, 1979). A problem with the method is that the cell traction force measurements generated are from all of the cells in the disk and not individual cells (Lo *et al*, 2000).

The second method involves the placement of fibroblasts onto a thin silicon membrane. When the fibroblasts apply cell traction forces the silicon membrane becomes wrinkled (Harris *et al*, 1981). The wrinkles are then undone by a micro needle and analyzed. A problem with using a silicon membrane is that it utilizes a computationally intensive method that has not been developed yet to find the force generated by a single cell (Wang and Lin, 2007).

The third method uses a force sensor array to measure cell traction forces. The force sensor array consists of micro fabricated cantilever beams made through lithography (Galbraith and Sheetz, 1997). The cells are placed onto the cantilevers and force is measured when they are loaded. The problem with the force sensor array method is that the cells can only attach to

discrete points on the micropillar, which does not mimic the *in vivo* mechanical environment. Moreover, the stiffness of the micropillar cannot be adjusted low enough to mimic soft tissues in the body.

In summary, a new method needs to be developed that allows for: single cell measurements, force measurements in all directions and real-time measurements. The new method should also have a high enough resolution for accurate results as well as be able to mimic *in vivo* conditions.

Chapter 3: Project Strategy

3.1 Initial Client Statement

The initial client statement provided for our project states:

The goal of this project is to develop a reliable method to fabricate hydrogels to allow measurements of traction force of living cells in real-time. Additionally, you will determine whether varying the stiffness hydrogels affect the traction force of NIH/*3T3 fibroblasts.*

The client statement does not include specifications. The initial statement also gives a method how to verify if the designed method functions through the use of NIH/3T3 fibroblasts. Through background research, analysis of the initial client statement, and clarifying the objectives of the problem statement a revised client statement was developed.

Noting that the problem statement deals with producing a method that allows for real-time measurements of cell traction force is important to the project. Also the problem has two objectives already in the initial client statement being able to fabricate hydrogels reliably and the ability to measure traction forces using the method.

3.2 Objectives

The objective tree below was created to help the team identify the hierarchy of needs and desired properties of the design. We categorize our objectives into three major groups: accuracy, reliability and user-friendliness. A more in-depth discussion of the objectives is included in the following paragraphs.

- Cell Traction Force Microscopy
    - Accurate
        - Relatively high resolution
        - Mimics *in vivo* environment
            - Smooth substrate surface
            - Mimics tissue stiffness
            - Provides nourishment and growth factors
    - Reliable
        - Sensitive measurements
        - Multiple measurements
            - Does not damage cell
            - Cells can be put in incubator and reused
            - Cell does not have to be removed
            - Provide reference to allow comparison between load and relaxed gel conditions
        - Reproducibility
        - Precision
    - User Friendly
        - Easy fabrication through protocol
        - Easy to handle

Under accuracy, our product is expected to have a relatively high resolution. As discussed before, the cell traction force microscopy (CTFM) relies on the tracing of fluorescent beads to measure the deformation the cell generates on the gel surface. The resolution of the measurement can be manipulated by adjusting the bead diameter and density to optimum values. A higher resolution is also considered one of the advantages of cell traction force microscopy over the micro-pillar array method. Therefore, our product needs to have similar resolution as obtained from the conventional cell traction force microscopy method.

Besides resolution, under accuracy, one key objective of the design is that the product needs to mimic the *in vivo* environment for the cells. Because our method is used *in vitro*, in order for the measurement results to be meaningful for medical applications, it is important that it presents to the cells a similar environment as in the body. The *in vivo* behavior of the cells can then be predicted from the *in vitro* tests. To be more specific, to mimic the *in vivo* environment includes three sub-objectives. First, we should provide the cells with a smooth substrate surface on which the cells can adhere to. The substrate surface will be coated with protein to create structure similar to the extracellular matrix found in body. Second, the hydrogel surface we fabricate need to have relatively low stiffness that mimics the body's soft tissues. The stiffness of the hydrogel must be controlled and should not vary randomly. Third, our product has to provide the cells with nourishment and growth factors for them to behave normally.

Under reliability, the first objective is that our method presents sensitive measurements. This is especially important for the success of the design because the force cells generate on the substrate is minimal, usually ranging from 20-200 nN. (Yu *et al*, 2012). Thus, our method has to be considerably sensitive in order to measure the minimal deformation.

Next, our design is expected to provide multiple measurements within the same sample and the same cell, under different conditions. This is the greatest challenge of our project and what makes our method special compared to the well-established conventional cell traction force microscopy. As discussed previously, in order to measure the deformation a cell generates on the gel surface, the old method requires the removal of the cell so that the fluorescent bead distribution under load and relaxed conditions can be compared. This limits the application of cell traction force microscopy because one sample is capable of only single time-measurement.

Our "Real-time" method needs to allow multiple measurements, without damaging or removing the cell; neither should it interfere in any aspects with any cellular activities, such as actomyosin activation. In order to fulfill such a goal, the device should provide a reference that allows the comparison between the bead distributions under load and relaxed gel conditions while the cells continue to exert force on the surface.

In addition, it is difficult for the microscope work stage to provide a suitable environment for the cells. The most important problem is that the room temperature, usually around 20 °C is considerably lower than the ideal 37 °C for cell culture. Together with other factors such as low CO_2 concentration and moisture, such problems create substantial challenges to keep the cells healthy and robust during a long-term experiment. Thus, our product should allow researchers to put the sample back in the incubator during intermission of an experiment and to continue the experiment afterwards.

The next objective under reliability is that our method has to be reproducible, which means by following an established protocol, fellow researchers should be able to replicate our product with the same quality. It also requires the method to be relatively free of unintended

variation and the same product can be produced every time during sample preparation. Lastly under reliability, the fourth objective is precision. We want our product to produce precise measurement results, presenting small or no variations with the same measurement.

The final group of objectives is user friendliness. It includes two objectives in particular. First one is easy fabrication through protocol. The method should not be excessively time-consuming. We expect to make the procedure as intuitive as possible to save the time researchers spend on sample preparation and shorten the cycles of experiments. The second one is ease to handle, which refers to when a researcher is to conduct an experiment with our product, he/she can start directly from seeding cells on the sample, and then put the sample under microscope to begin the experiment. The standard here is to compare with the conventional cell traction force microscopy. We want our method to involve very few or no extra steps before or during experimentation and data analysis.

In order to rank and rate the relative importance of the objectives, the team created a pair-wise comparison chart to compare all the objectives under the second level in the objective tree. The chart can be found in Appendix 1. Here is the final ranking of the totals for each objective

1. Mimics *in vivo* environment/ Multiple measurements
2. Reproducible
3. Sensitive measurements
4. Easy fabrication protocol/ Precise/ Relatively high resolution
5. Easy to handle

The ranking generally agrees with the group members' expectations. Mimicking the *in vivo* environment and allowing multiple measurements are both the most critical aspects for success

of our design. Reproducibility is also substantial because our project is more about developing a method than creating a product, and only when the results of the method can be replicated accurately can the method be concluded successful. Sensitive measurements, precision and high resolution are rated as relatively low ranking because they are the desired features for the product instead of absolute needs. Lastly, because our project is research-based instead of market-based, the two objectives under the user-friendliness group is ranked as the lowest given the priority of other objectives.

3.3 Constraints

List of constraints:

- Budget (~$458)
- Time (Beginning of D Term)
- Safety (Safe for users)
- Size constraints (Must fit in incubator and under microscope)
- Should not damage cells or interfere with natural functions
- Testing limited to *in vitro*
- Fluorescent beads must be visible under microscope
- Hydrogel mechanical properties must remain constant during preparation and use

Some common constrains that will need to be addressed in the project will be the budget, time period, and safety of the method. A more specific set of constraints that affect only our project are: size constraints, maintaining cell health, *in vitro* testing, fluorescent bead visibility, and maintenance of hydrogel mechanical properties. Each issue will have to be addressed differently and be taken into the design process to limit the design space.

The issues of budget which is around $458 will be dealt with by minimizing the usage of expensive reagents. Another way of dealing with the budget will also be to seek out cheaper alternatives if possible and not wasting any reagents. Time is also a common constraint which can be dealt with by meeting deadlines and planning ahead.

Safety of the user is also an important constraint. The method should not deal with highly toxic chemical reagents and if it does the method should have a proper protocol for how to handle and manage the reagents. The safety of the user when performing the method has to be taken into account since the method will be performed in a laboratory setting.

Size constraints in the case of our project will mainly be dealing with incubation and microscopy. The product should fit into a standard incubator. Also using the method under a microscope means that it must be small enough to fit on a microscopy stage and fixed in place. The method will have to be either petri dish sized or smaller.

Cell health is also important to our method. The cells undergoing the process of being put on a gel and not being taken off should not undergo any damage. The reason cell health is important is due to the accuracy of the results, if cells are damaged the cell traction force results will be incorrect. Maintaining cell health will involve using standard cell culture practices, medium, and minimizing the usage of unknown chemical substrates that can cause unknown affects. Another factor that is part of maintaining cell health is that the method should not interfere with normal cell contractions on the gel.

Keeping the mechanical properties of the hydrogel constant during preparation and usage will also have to be taken into account. Having a varying stiffness through time of the hydrogel can cause incorrect results to be reported. One way of dealing with this would be to have a

standard procedure that can be used when fabricating the polyacrylamide hydrogel. Storing the hydrogel with the correct amount of moisture will also be a factor in achieving constant mechanical properties.

The fluorescent beads placed on the hydrogel also have to be visible under the fluorescent microscope. The reason fluorescence is a constraint is because putting to many beads or the wrong type can cause the image taken to become hard to view. If the image cannot be seen clearly then the computational results will not be accurate. Using the correct fluorescent materials with the correct concentrations and a clear transparent hydrogel can regulate the fluorescence visibility.

Testing of the method is constrained to *in vitro* since human test subjects cannot be used to measure cell traction force. The method proposed should mimic *in vivo* conditions to yield accurate results. The usage of NIH/3T3 fibroblasts to verify the results will also work towards mimicking an *in vivo* model.

3.4 Revised Client Statement

The revised client statement with specifications:

- *To monitor cellular activity during culture, it is important to characterize the cell traction force (CTF) in real-time*
- *Develop a reliable method to fabricate hydrogels to allow measurements of CTF in real-time without damaging or interfering with the cells' actin activity*
- *Allow multiple measurements on a single cell to be taken over long periods of time.*

- *The hydrogel must provide a substrate for cell adhesion and cell contraction as well as having a controlled stiffness.*
- NIH/*3T3 fibroblasts will be used to verify the feasibility of the method*

Comparing the initial client statement to the revised client statement reveals the details of the problem statement. The revised client statement makes the objectives of the project clearer. Some objectives that result from the revised client statement are having a controlled stiffness, specifications and a range for force measurements, and the original goal having the method be real time.

The revised client statement highlights the major functions of the design:

- Embed fluorescent beads on top surface of hydrogel.
- Allow cell adhesion to gel surface.
- Allow measurements of cell traction force under microscope.
- Enable real-time measurements.

3.5 Project Approach

In order to meet our constraints and objectives, the approach in general is to develop a method to fabricate polyacrylamide hydrogel with fluorescent beads embedded on the top surface. Below is a detailed discussion about our approaches to address each specific constraints and objectives.

3.5.1 Approach to Constraints:

For the budget constraint, we should utilize the resources currently available in Prof. Wen's lab in prior and purchase new ones only when necessary. The project in general is not expected to involve expensive chemicals, and therefore the budget is not a major concern. For the time constraint, it requires the team to manage the limited time well. A project timeline and plans for B, C and D terms should be set up before the end of A term. In terms of safety concerns, we will minimize involvement of hazardous chemicals and all of our procedures will follow general lab safety regulations. The product from our method will be fixed on a standard petri dish in order to meet the size constraints. The petri dish allows the cells to be incubated in the incubator and fixed in place under microscope work stage.

The key constraint relating to the real-time measurement provided by the product is that it should not damage the cells or interfere with the cells' natural functions in any ways. Therefore, we will use standard cell culture medium and minimize the use of chemicals with uncertain effects. Any potentially toxic chemicals involved in the fabrication of the hydrogel should be eliminated.

Because the testing of the method is limited to *in vitro* testing, we will use NIH/3T3 fibroblast for the *in vitro* testing of the product. Studies on cell traction force have been done extensively on this specific cell line. As a result, using NIH/3T3 fibroblasts allows us to compare our results with results from the well-established methods so that we can evaluate the accuracy provided by our method.

Next, our fluorescent beads embedded must be clearly visible under microscope. In order to meet this constraint, strong fluorescent materials will be used at an optimum concentration.

The hydrogel must also be transparent with minimum deflection of light. The use polyacrylamide hydrogel is expected to address such need since it is a widely accepted as the best choice for cell traction force microscopy.

In addition, the hydrogel should not degrade after preparation and its mechanical properties must remain constant before, during and after use. This requires the team to follow the standard procedure for fabrication of the polyacrylamide hydrogel. The gels should also be stored properly and kept moistened in controlled temperature.

3.5.2 Approach to Objectives:

The product from our method should be able to mimic the *in vivo* environment for the cells. By varying the concentration of acrylamide and the cross-linker, the stiffness of the polyacrylamide hydrogel can be precisely controlled, which allows the production of hydrogel with similar stiffness of the soft tissue in body. In order for the cells to adhere and exert force on the gel surface, the gel will be coated with collagen, which is one of the most abundant compositions of extracellular matrix and encourages the cells to behave similarly as *in vivo*.

The objective of multiple measurements requires the method not to remove or damage the cells. Therefore, the method will use only standard cell culture medium with no additional chemicals applied to the cells. There are two possible approaches to allow "real-time" measurement. First, we could create a geometric lattice of fluorescent beads utilizing the self-assembly tendency for charged particles. (Isa *et al*, 2010). If we manage to create a perfect lattice of fluorescent beads on the gel surface, any deformation generated by the cell will be identified and processed by computer software, therefore provides the most straight forward solution to real-time measurement. However, this approach can prove highly challenging because the self-

assembly ability of the fluorescent beads does not guarantee a perfect lattice as shown in previous studies (Isa *et al*, 2010). Considerable variations of the beads' individual locations exist and may present unexpected problems to cell traction force measurement.

Therefore, another proposed approach is to construct a reference frame for identifying the locations on the gel surface. In this way, the fluorescent bead distribution can be first imaged under a microscope before the cells are seeded which gives the researcher data of the relaxed condition beforehand. After the cells are seeded and during the experiment, the reference frame allows the researcher to identify the location of a cell on the gel surface, in order to compare the fluorescent image under load condition with the corresponding relaxed one.

The reference frame can be created with fluorescent beads of different color, density and diameter, or by other methods such as pre-drawn grid lines. This approach can be considered a detour to achieve the goal. It does involve extra steps to image the beads before experiment for every sample. However, it may prove to be a more viable option, and therefore yield better accuracy and reliability than the lattice method.

The two approaches will both be pursued and tested to evaluate their pros and cons. The team will decide the specific one to choose after a comprehensive comparison of the two. Moreover, it is also possible that the final method we develop incorporate both approaches.

In order to address the objective of reproducibility, the method should minimize involvement of undefined factors. Every procedure needs to be carefully recorded and any arbitrary variation from manual operations has to be eliminated. The method will also be tested extensively to present statistically stable results, so that we ensure that the result is controlled and reproducible.

For the objectives relating with the quality of measurements, which are sensitivity, precision and high resolution, the team's approach is primarily from careful manipulation of the fluorescent bead distribution. The diameter and density of the fluorescent beads affects the quality of the measurements directly since the measurements are conducted by tracing the displacement of the beads. An optimum value for diameter and density of the fluorescent beads should be selected based on mathematical calculations as well as empirical testing.

Last but not the least, in terms of the two objectives under user-friendliness, easy to fabricate and easy to handle, our method is expected not to involve any complicated techniques. The materials and equipment used should be easily accessible for most labs in the country. Therefore, the team is going to primarily focus on utilizing the facilities available at the Gateway Park labs. The final protocol for the method ought to be comprehensive and intuitive as well.

Chapter 4: Design Alternatives

4.1 Functions and Specifications

The function of the method for measuring real time cell traction forces involves various components. The functions the design must follow involve:

- Be able to embed fluorescent beads on the top surface of the hydrogel,
- Vary stiffness of the hydrogel
- Assess the distribution of the beads on the gel.
- Design must also enable cell adhesion to the gel surface
- Allow measurements of cell traction force under a microscope
- Enable real time measurements.

The first function of being able to embed fluorescent beads in the top layer of the surface of the hydrogel is vital to reading cell traction force measurements. The reason for beads being placed on the top surface is due to the cells being placed on the top layer of the hydrogel and applying force at the top most layers. 95% of the fluorescent beads should be embedded within a 10 μm thickness from the top to ensure accurate results. The thickness distribution can be examined under confocal microscope.

Being able to vary the stiffness of the gel is also a vital function of the design. The reason for varying the stiffness of the hydrogel is due to the correlation between cell traction forces and substrate stiffness that has been observed in previous research. (Wells, 2008) Being able to vary the stiffness of the hydrogel throughout multiple samples also allows for measurements to be taken in conditions that mimic an *in vivo* environment. The stiffness of hydrogel should be in a range from 2 kPa – 20 kPa similar to the range of soft tissues in the body (Yu *et al*, 2012). Being able to assess the fluorescent bead distribution of the hydrogel is also relevant to obtaining accurate cell traction force measurements.

Assessing fluorescent bead distribution throughout the hydrogel allows for more accurate cell traction force measurements. An optimum bead density is required for high quality measurements. The density should also be uniform throughout the gel surface. In addition, aggregates of beads need to be avoided because they may skew the results.

Cell adhesion enables cell traction force measurements. Without proper cell adhesion or focal adhesions as mentioned previously, cells will not be able to contract. Enabling cells to attach to the hydrogel through the usage of collagen allows for cell traction forces to take place.

Another function that is vital to the overall design is being able to take cell traction force measurements under a microscope.

Establishing a well-defined method for measuring cell traction force measurements is also a major aspect of the design. The reason for making a clear and concise method is due to reproducibility and the end user side of the method. The cell traction force range using our method should be from 0-100 nN (Yu *et al*, 2012).

Enabling measurements through images taken under a microscope means that different types of cells can be analyzed due to the previous function of substrate stiffness is variable in different samples. To verify our method NIH/3T3 fibroblasts will be used since they play a key role in many physiological processes such as wound healing and have been previously researched (Munevar *et al*, 2001).

Finally the most important function of the design is to enable real-time measurement, where the cell does not need to be removed from the hydrogel for cell traction force measurements to be taken. The reason this is vital to the design is because currently there is no method established to take real time cell traction force measurements on deformable polyacrylamide hydrogels.

Our team came up with four design alternatives to enable real-time CTFM:

- Self-Assembling Lattice Method
- Reference Bead with Protein Grid Method
- Bead Projection Method
- Micropillar Method

Each method utilizes different components to achieve the same goal. The function means chart in Appendix 2 summarizes all four alternative designs.

The Self Assembling Lattice method involves the usage of a liquid-liquid interface (Isa *et al*, 2010). Fluorescent beads are injected into the liquid-liquid interface between water and hexane (Fig. 4-1a). A glass slide is then dipped into the interface and lifted out. As result, a lattice structure of beads is deposited on the glass (Fig. 4-1b). The lattice can then be transferred onto the hydrogel surface (Fig. 4-1c). A computer generated lattice can then be compared to the actual lattice image once a load is applied by a cell (Fig. 4-1d). The comparison yields more accurate cell traction force measurements and the cell is not required to be removed.

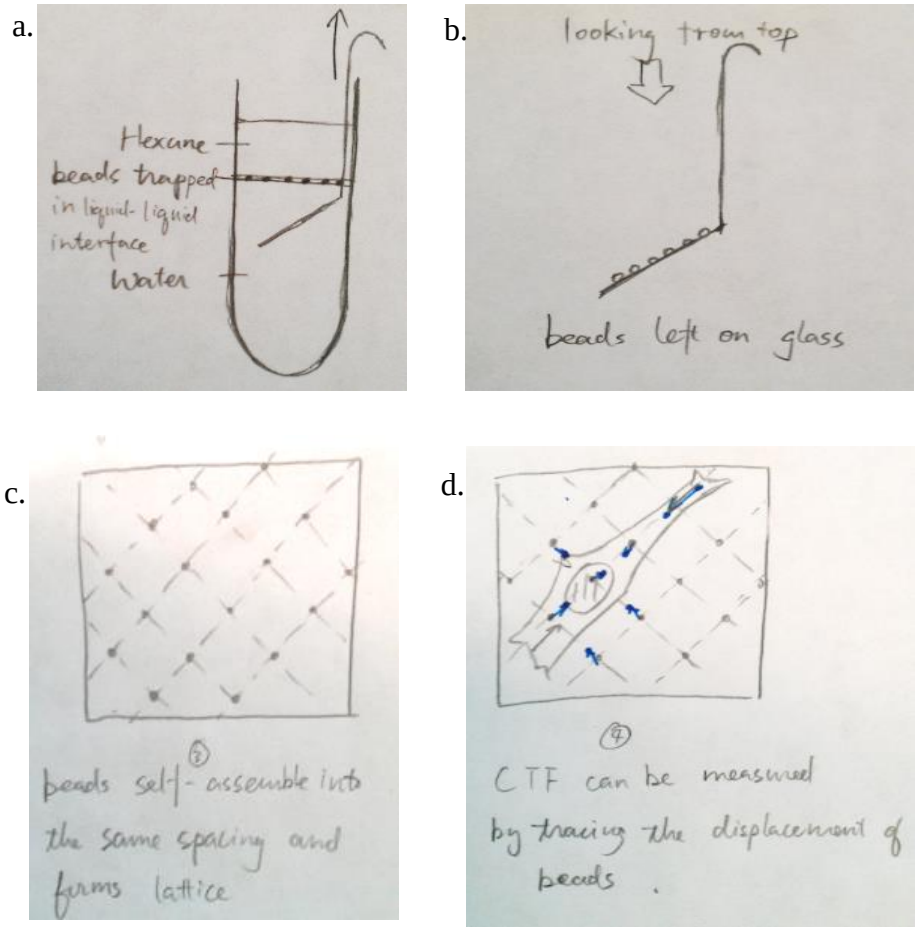


Figure 4-1: Schematics for Self Assembling Lattice Method

The Reference Bead with Protein Grid method uses two types of fluorescent beads to obtain cell traction force measurements. Types of beads with different diameters and fluorescence (green and red) are mixed together (Fig. 4-2c). The beads with a larger diameter are embedded into the hydrogel at a lower concentration. The larger diameter green beads can be used as a location indicator under the microscope while the smaller red beads are used to trace the deformation of the gel surface for cell traction force measurements. The protein grid can be considered a second component that is combined with the reference bead method.

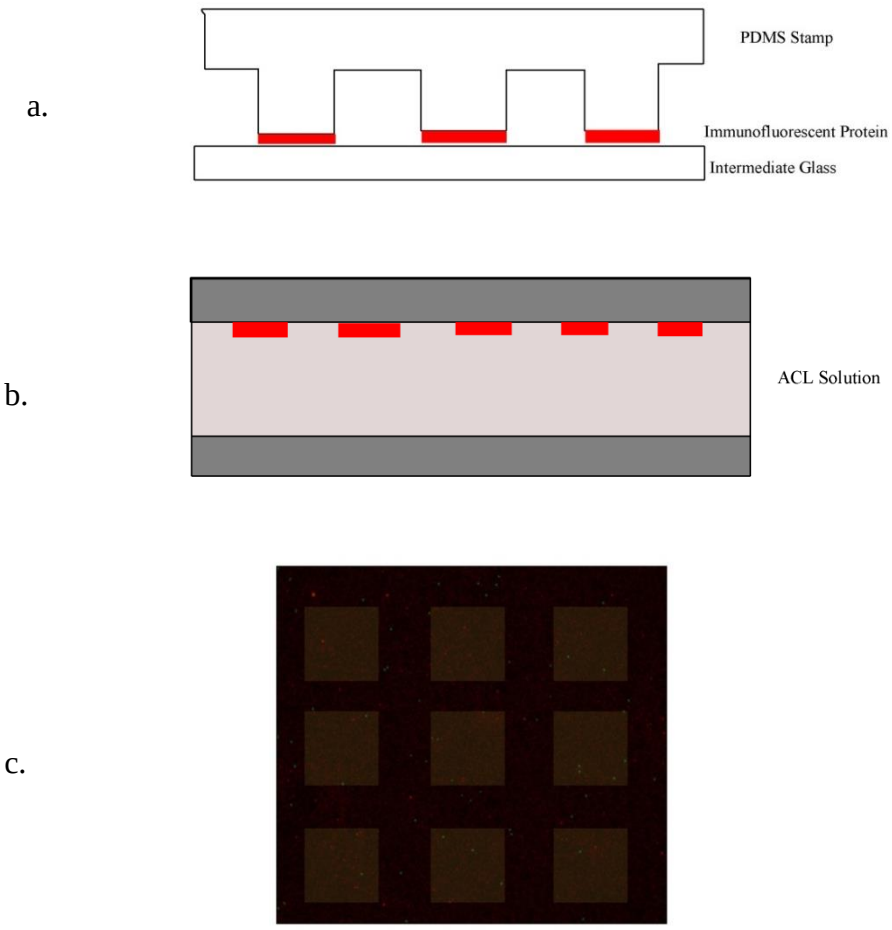


Figure 4-2: Schematic of Reference Bead with Protein Grid Method

The Bead Projection method uses the development of a photosensitive coating to acquire cell traction force measurements. In the bead projection method a photosensitive coating is placed on the bottom of the hydrogel. Then a light is shined vertically from the top of the gel to develop an image of the beads onto the coating (Fig. 4-3). After the cells are seeded and adhere to the surface, the loaded image can be compared to the developed bead pattern on the coating layer for calculating cell traction forces.

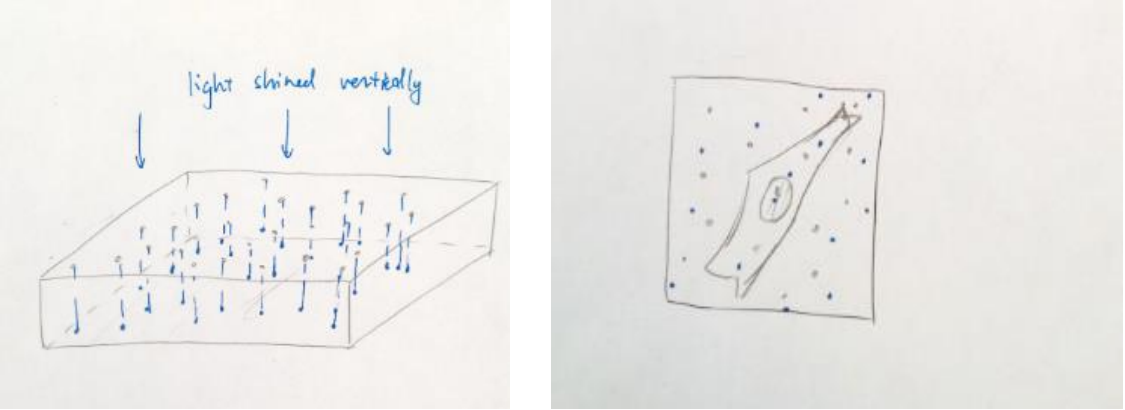


Figure 4-3: Schematic of Bead Projection Method

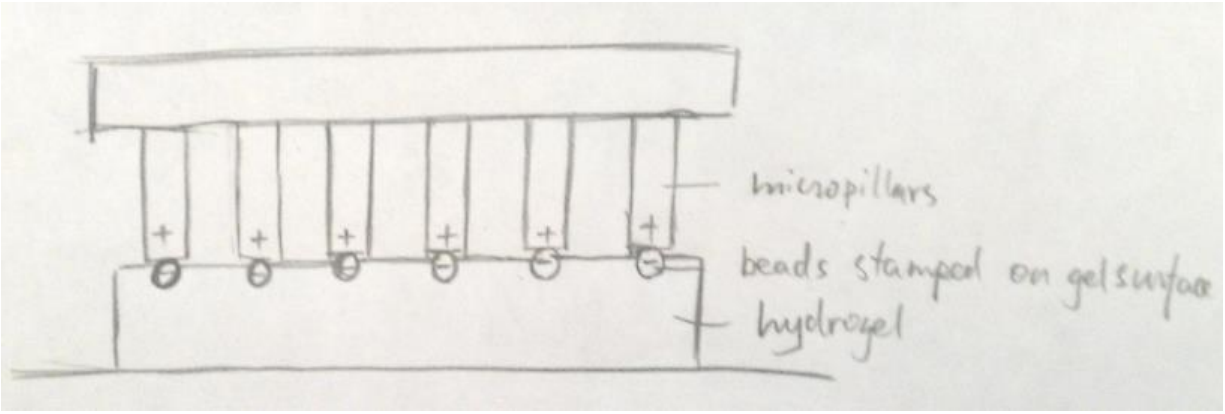


Figure 4-4: Micropillar Method

Finally, the Micropillar method involves using micro-fabrication techniques to produce an array of micropillars (Olivia *et al*, 2005). The micropillars can be dipped into fluorescent beads and stamped into the polyacrylamide hydrogel (Fig. 4-4). The stamping of the beads will make an array. The cells can then be placed onto the hydrogel causing a load force. The array can be compared to its original state and the cell traction force can then be calculated.

4.2 Conceptual Tentative Final Design

In order to make our decision for the final design among the four alternatives, we created an evaluation matrix, which can be found in Appendix 3. The evaluation matrix includes three specific constraints about the project and four key objectives. According to the evaluation matrix, all four design alternatives had considerably high scores in meeting the objectives. Among the four, reference bead and micropillar methods received the highest scores.

However, certain limitations exist in all four alternatives. For the self-assembly lattice method, the reproducibility is questionable, because the reliance on the self-assembly capability of charged particles does not always result in a perfect lattice geometry. Random offset of beads within a range of 5 μm was observed in the previous study (Isa *et al*, 2010). Such offset could considerably affect the accuracy of cell traction force measurement if relied only on the comparison with a computer generated lattice.

For the bead projection method, it is uncertain if the constraint of bead visibility is satisfied, because the extra coating at the bottom may disturb the light path for the microscope. A more crucial problem occurs in reproducibility. Due to the interference, refraction, and diffusion of light in a microscopic scale, it is extremely challenging to develop the pattern of the beads

onto the photosensitive layer. The method lacks proof of concept and therefore the feasibility is highly questionable.

Although the micropillar method receives the highest score for the objectives, its cost may not conform to the budget constraints. The fabrication of the micropillar mold requires an expensive technique called silicon etching. The cost is especially high for our case because we want to make the micropillar array at sub-micrometer level (Olivia *et al*, 2005). Before further research is done to estimate the cost, we are unsure whether our budget permits the fabrication.

As a result, the reference bead method is selected as the tentative final design at the current stage. It meets all the constraints and receives high scores on all the objectives except for user-friendliness. The reference bead method requires imaging the entire gel surface before the experiment. This extra step in experiment preparation can be time-consuming, but given the priority of other objectives, this limitation does not significantly weaken the advantage of the method.

The technical details of the tentative final design include four major components:

1. Polyacrylamide (PA) hydrogel fabrication with embedded fluorescent beads
2. Micro-patterning grid stamped on the bottom of hydrogel
3. Self-assembly of beads in liquid-liquid interface
4. Data analysis using Particle Image Velocimetry (PIV)

The first component is the key of our final design. Polyacrylamide hydrogel is used extensively in cell traction force studies. It has several advantages compared to other materials. Despite the fact that the PA gel is a viscoelastic material, its stiffness does not change significantly with deformation within a certain range (Wang and Lin, 2007). It is also very easy

to fabricate. In addition, the stiffness of the PA gel can be easily controlled and varied by changing the concentration of acrylamide and the cross-linker N, N'-methylene bis-acrylamide (bis) (Tang *et al*, 2012). Once fabricated, the PA gel's properties are highly stable for an extended period of time, which gives long shelf life to our product. Furthermore, the PA hydrogel can be coated with various proteins such as collagen, laminin and fibronectin to allow cell adhesion

Fluorescent beads are embedded on the top surface of the hydrogel during the polymerization. The addition of fluorescent beads does not change the properties of the hydrogel and allows the tracing of the deformation generated by cell traction force. This enables the cell traction force to be calculated based on the displacement of fluorescent beads and the known stiffness of the gel. To be more specific, two types of micro beads with different colors and diameters will be mixed in different concentrations. For example, a set of red beads with smaller diameter is used for measuring the deformation and a set of green beads for position indicator. The two types of beads can be imaged separately under different fluorescent filters.

Micro-grid is used to provide a general location indicator. Due to the fact that a certain location needs to be identified accurately from the entire gel surface, the sole reliance on reference beads is time consuming even for a computer program because the search area is large. By creating a 20 by 20 block grid coordinate system on the hydrogel with the grid pattern stamped on the gel surface, we are able to reduce the size of interest by 400 times. Therefore, when the experiment is conducted, the researcher needs to first identify and record the x and y coordinates of the grid being observed. As a result, the process of location identification with reference beads is dramatically faster.

Moreover, we would like to incorporate the self-assembly lattice into this method because the lattice yields better measurement quality than random bead distribution. The self-assembly of fluorescent beads is conducted by injecting fluorescent beads into a liquid-liquid interface between water and hexane (Isa *et al*, 2010). Because the beads all have the same mass and the same negative charge, the repulsive electro-static force arranges the distribution of the beads into a lattice with the same spacing between every two neighboring beads. However, due to the limitations on reproducibility discussed above, we will still compare the image of the lattice under cell contraction with the image of unloaded state instead of computer generated geometry. Further tests will be conducted to evaluate if the lattice generated is superior to random bead distribution, based on which the team will decide whether or not to incorporate this approach.

Data analysis is done by tracing the displacement of fluorescent beads using Particle Image Velocimetry (PIV). It is used extensively in relevant studies (Wang and Lin, 2007). It generates a two dimensional displacement vector map by comparing the images of the beads with and without deformation, from which the cell traction force exerted on the gel surface can be calculated.

4.3 Feasibility Study and Experiment Methodology

In terms of proposed feasibility, all four major components as listed above have convincing evidence to attest their feasibility, either from the research undergoing in Prof. Wen's lab or published studies from other groups.

The PA gel fabrication with fluorescent beads embedded is a well-established method used in conventional cell traction force microscopy. In Prof. Wen's lab, one of our team members has already conducted numerous CTFM experiments using the method. It is well-

controlled in all steps involved, and the results are highly reproducible. The PIV analysis has also been conducted on the data and has yielded satisfactory results. Therefore, we have sufficient evidence for its feasibility.

The self-assembly of fluorescent beads is a method reported in a previous study done by other groups (Isa *et al*, 2010). In the article, they showed images of the fluorescent bead lattice with relatively high quality. Statistics on the distribution of the beads and their offset was also provided. According to the paper, the self-assembly at liquid-liquid interface is a reliable and precise approach (Isa *et al*, 2010). The method and materials are described in details in the article. It does not involve the use of any complicated technologies and the materials used are readily available in Prof. Wen's lab. Thus, we are going to follow their protocol in order to replicate the results.

The way all these components work in one system is through the use of an intermediate glass for gel fabrication. First, fluorescent beads of two colors are deposited on the intermediate glass. Next, the protein pattern is stamped on the intermediate glass. The intermediate glass is then used to sandwich the PA solution with a functionalized bottom glass, treated with glutaraldehyde to allow the binding between the gel and the glass (Tang *et al*, 2012). As the polymerization of the PA gel occurs, both the fluorescent beads and the protein pattern are transferred onto the gel surface. All these different steps are expected to function separately and not interfere with one another.

The proposed experiment to test the final product of the method is to apply the method directly to real-time cell traction force microscopy. A large area of the hydrogel surface will first be imaged under a fluorescent microscope and the images saved in the computer. Next, NIH/3T3

fibroblast cells will be seeded at relatively low density (~10% confluency) onto the hydrogel and incubated overnight in a cell culture incubator to allow the adhesion of the cells.

The next morning, the hydrogel will be placed under a microscope and several cells will be selected for traction force measurement. The microscope we use allows saving the relative x, y and z coordinates of the location on a control stage, which allows us to measure multiple cells on a single piece of hydrogel. The cells to be selected need to be healthy in morphology and separated from other cells by a certain distance. Both the tracing beads and the reference beads will be imaged separately on the location of each selected cell.

After imaging, the samples are put back into the incubator. The location of each cell is recorded. In order to test the accuracy of location indication, multiple measurements can be conducted afterwards by placing the sample back under the microscope to find the location based on the previous records.

After obtaining data from multiple measurements, the images will be compared with the original images of the unloaded state taken before the experiment. The magnitude of cell traction force exerted will be compared to statistical results from unpublished data in Prof. Wen's lab on the cell traction force under the same conditions measured by conventional CTFM. Because the conventional CTFM is well-established, if our measurement results agree statistically with the previous data, we can conclude that our new method has the desired accuracy.

4.4 Preliminary Data

The team has conducted several tests for the proof of concept. The results are generally promising, as described more in depth below.

4.4.1 Deposit and Transfer of Fluorescent Beads

An evaporation method was used to deposit the beads on the intermediate glass in a random but uniform distribution. The fluorescent beads (InvitrogenTM Lot: 1010102) were suspended in ethanol Ethanol (Fisher Scientific, Lot: 112499) at 0.5 wt.% concentration. The intermediate glass (Corning, Cat#: 2865-25) was treated with oxygen plasma to make it highly hydrophilic. Fifty microliters of the bead suspension was dropped onto the glass and allowed to spread uniformly across the glass surface by virtue of the high hydrophilicity introduced through plasma treatment. The glass was quickly placed on a heater cube and heated at 150 ^{o}C under 20 mmHg vacuum. The high temperature allows for fast evaporation of the liquid, leaving the fluorescent beads attached to the glass without aggregates. The intermediate glass was then used with the functionalized glass to sandwich the acrylamide solution (8% acrylamide, 0.1% bisacrylamide) (Bio-Rad Cat# 161-0140, 161-0142). After polymerization, the intermediate glass was carefully peeled off. The hydrophilicity of the intermediate glass allowed complete transfer of the beads onto the gel surface as no beads were observed on the intermediate glass after separation. The image of the red fluorescent beads on the gel surface under 40x objective is shown below. (Fig. 4-5) The density of the beads was the desired density for cell traction force measurements, and was uniform throughout the gel surface.

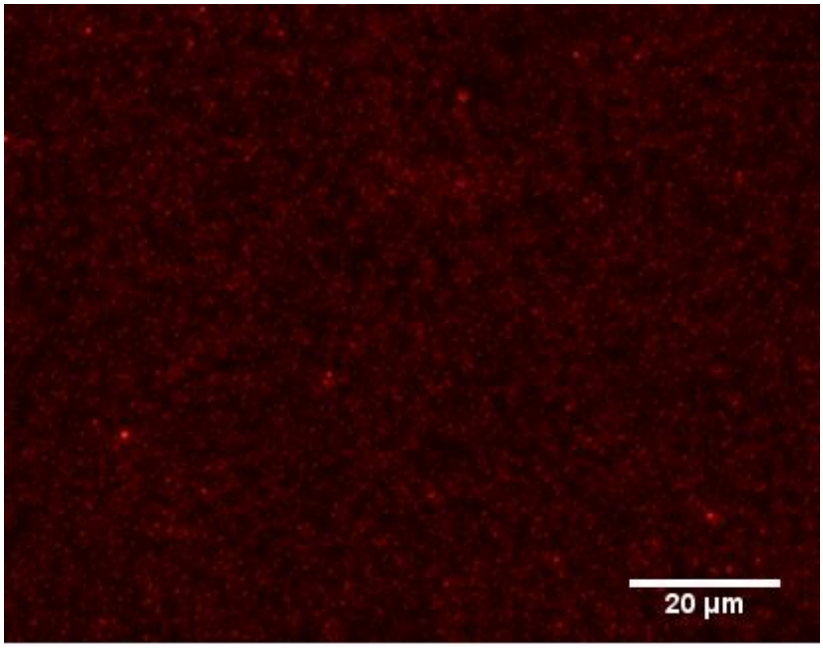


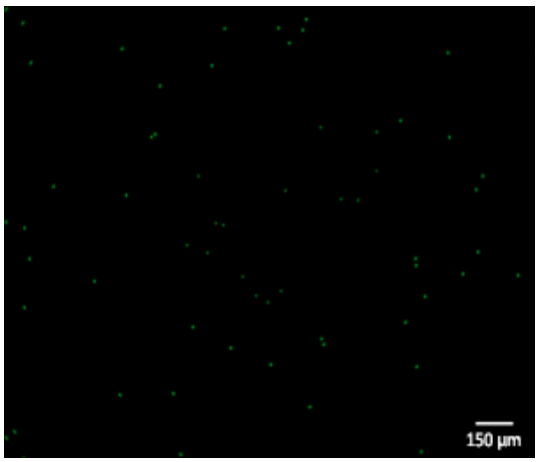
Figure 4-5: Bead distribution on the gel surface under 40x objective

4.4.2 Mixture of two color beads

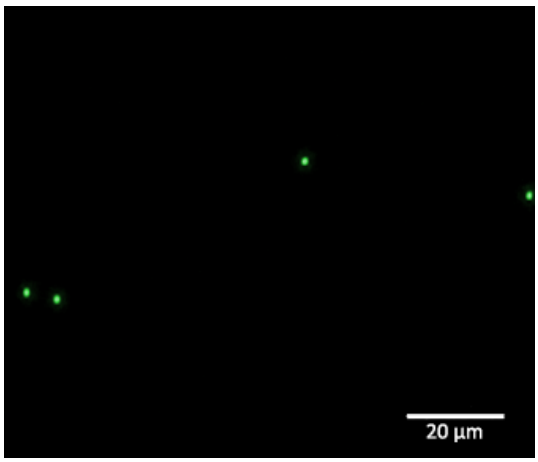
In order to test the feasibility of mixing fluorescent beads of two different colors, we conducted another test. The same red fluorescent beads with diameter of 0.2 µm (Invitrogen^{TM} , Lot: 1010102) as used in the first experiment above were mixed with green fluorescent beads with diameter of 2 µm (Invitrogen^{TM,} Lot: 906909). The green beads serve as the reference location indicator. The beads are once again suspended in ethanol at different concentration (0.5 wt.% for red, and 0.05 wt.% for green). The same methods for bead deposition and gel fabrication were used.

The images showing the bead distribution can be found Fig.4-6. The results showed that the mixing of two types of beads does not interfere with the random distribution of the two beads. Both beads were perfectly visible under different filter channels and the image quality

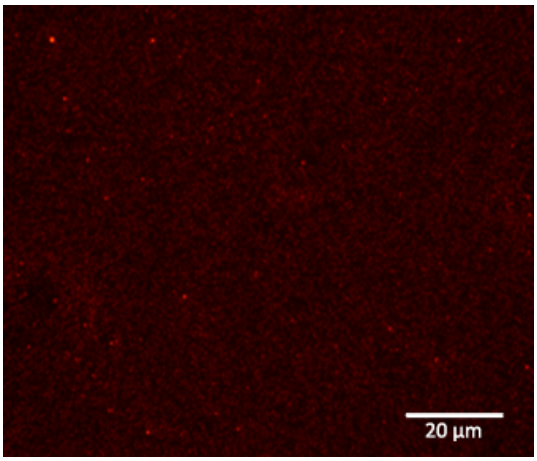
was not affected by the mixing. A low concentration of the reference beads gave a sparse distribution, from which identification of location through computer program is possible.



a. Green bead, 10x objective



b. Green bead, 40x objective



c. Red bead, 40x objective

Figure 4-6: (a) Green Bead (10x), (b) Green Bead (40x), (c) Read Bead (40x)

4.4.3 Collagen coating and cell adhesion

For this test, collagen was coated onto the PA gel surface directly. The PA gel surface was coated first with a layer of Sulfo-Sanpah (GBiosciences®, Cat#: BC38), which binds to the PA gel on one end and binds to the collagen on the other. Typy-I collagen (BD Biosciences, Lot: 11093) solution at a concentration of 50 μg/mL was dropped at 250 μL per gel on the gel surface with a size of 2.5 x 2.5 cm. Depending on the interested ligand, other proteins such as fibronectin (20 μg/mL) and laminin (100 μg/mL) can also be used. After 3 hours, the excessive collagen solution was rinsed off. NIH/3T3 fibroblast was then seeded onto the gel surface at 10% confluency. The sample was incubated overnight at 37 °C to allow adhesion of the cells. After incubation, the cells on the gel were observed to elongate and adhere to the gel surface. The cells maintained normal and healthy morphology, as shown in the figure below (Fig.4-7). These results indicate that the collagen coating allows the adhesion of cells on the gel surface and the system mimics the cells' *in vivo* mechanical environment to a certain extent.

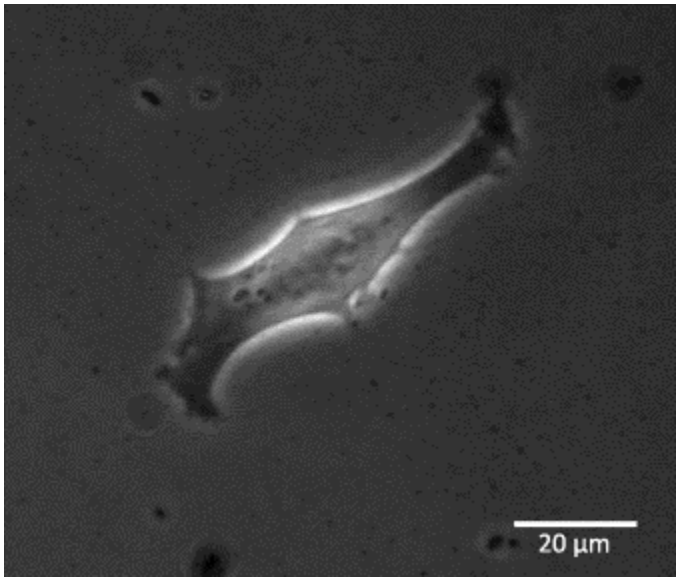


Figure 4-7: Phase contrast image of NIH/3T3 fibroblast attaching to the gel surface, under a 40x objective

4.4.4 Cell Contraction on the PA Gel

In order to test whether or not the NIH/3T3 fibroblasts were exerting force on the gel surface, we continued the experiment based on the protocol for conventional CTFM. The fluorescent image of the beads was taken when the cell adhered to the surface (Fig .4-8).

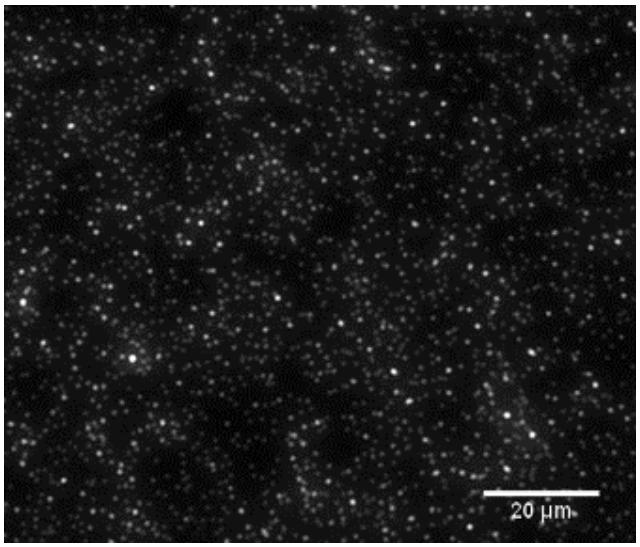


Figure 4-8: Fluorescent beads at loaded condition, 40x objective

The medium was then aspirated and PBS was applied to rinse the sample. Next PBS was aspirated and 2.5% trypsin solution (Gibco®,Lot: 25300) was applied to the sample in order to remove the cells from the gel surface. After 3 minutes, the cell was no longer attached to the surface. Another image of the fluorescent beads was taken (Fig. 4-9). A comparison of figures 4-8 and 4-9 indicates the integrity of bead conformation before and after trypsin treatment.

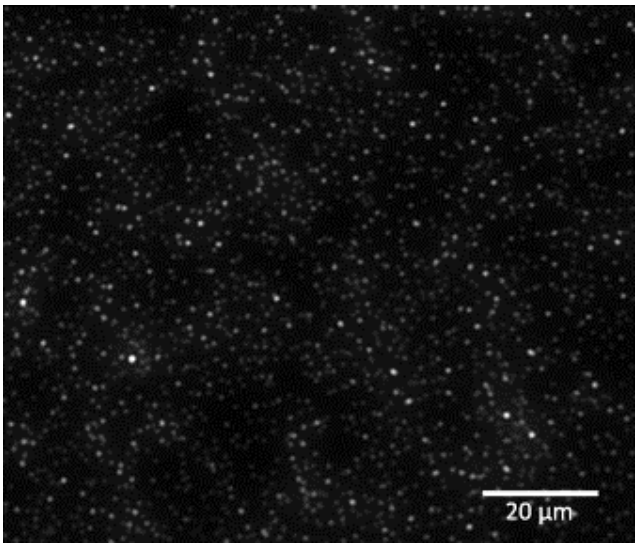


Figure 4-9: Fluorescent beads at relaxed condition, 40x

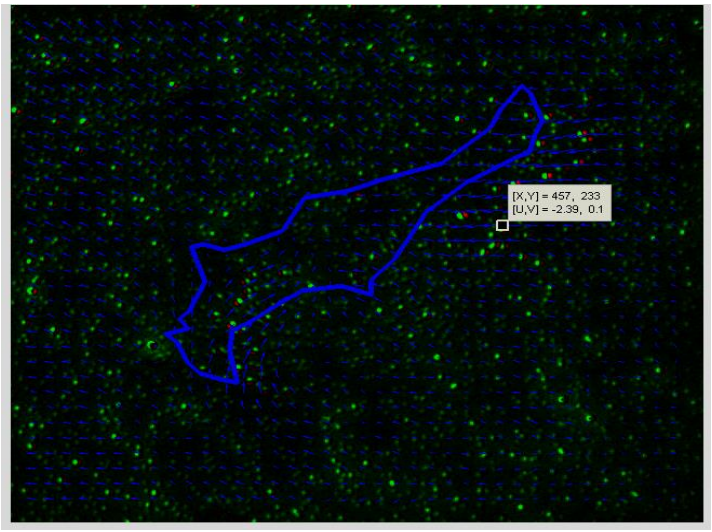


Figure 4-10: Displacement vector map generated by PIV analysis

By comparing the two images using PIV, a displacement vector map was generated (Fig. 4-10). As we can see from the image, around the outline of the cell, there is significant displacement of the fluorescent beads between the load and relaxed states. The vectors point inward to the cell body, which means the cell was applying force on the hydrogel surface. This experiment serves

as a very good proof of concept for measuring the cell traction force by tracing the bead displacement.

4.4.5 Self-assembly bead lattice

Method:

1. Preparation of bead solution: 5% bead suspended in [6:4 DI water: ethanol] and sonicated for 10 minutes
2. Liquid-liquid interface made in 50 mL centrifuge tube with Hexane (Fisher Scientific, Lot: 121604) and DI water
3. Insert glass and holder into interface
4. Inject bead solution with pipette tip. Volume of beads adjusted
5. Wait for equilibrium
6. Lift holder and slider at constant velocity (25 micrometers/second)
7. Air-dry the glass

Modifications:

Many modifications were done to the self-assembling bead method including:

- Volume of bead solution injected
    - 2, 5,20, 30, 40,50 uL
- Rate of injection
    - Micropipette injection by hand
    - Syringe pump injection (rate of 0.5 microliter/min)
- Pulling rate
    - Pulley system operated by hand
    - Syringe pump motor configuration
    - Microscope pulley system

- Treatment of glass
    - Plasma treated glass
    - APTMS treated glass
    - Untreated glass
- Diluted vs. undiluted
    - Bead dilution (5%)
    - Undiluted bead injection
- Equilibrium time
    - 0, 5, 10, 15 minutes

Results:

Effects of volume injected

The volume of the bead solution injected affected the aggregation and pattern of the beads on the glass. At lower volumes of 2 and 5 microliters the beads were viewed under fluorescence microscopy and shown to have very few aggregations while having a sparse distribution not fully covering the glass. There was no lattice observed at low volumes.

In higher bead solutions (20, 30, 40, 50 microliter) there were aggregations of beads present. No lattice resulted. The beads at higher volumes had a randomized distribution and no lattice was observed. The volume of the bead solution is important to lattice formation due to the charge of the beads acting on each other. If the volume is too sparse then no charge interaction will take place whereas too much crowding can also overpower lattice formation.

Effects of injection rate

The rate of injection was studied using micropipette injection by hand and motorized syringe pump injection. The bead solutions were injected in initial tests by a micropipette into the liquid-liquid interface. Due to the density of the beads and the difference in the density of water (more dense than beads) and hexane (less dense) the fluorescent beads should have been trapped in the interface between the two. The beads were observed to first sink into the water layer and then float back up into the hexane and water interface. A syringe pump was used to inject the beads at a much lower rate (0.5 ml/min).

The syringe pump method reduced the amount of distance the beads would drop into the water layer before flowing back up to the interface. If the beads were injected into the hexane layer a droplet would form and eventually fall through to the water layer and eventually equilibrate at the interface over time due to the difference in density. Using each method did not yield a lattice like structure when tested.

Effects of pulling rate

The pulling rate of the glass slide was also studied using two methods: a pulley system and a syringe pump as a motor. The pulley system was set up using a pulley, and a string tied to the metallic holder that was composed of wire. The pulley system was tested using different pulling rates ultimately operated by hand. The pulling rates did not have a significant effect on the bead formation and was ultimately deemed unsuitable for usage since hand pulling rates are much faster than 25 micrometers per second.

The second system involved using a syringe pump configured to move at a rate of 25 micrometers per second. The syringe pump was used at an injection rate of 0.05 ml per minute which is close to 25 micrometers per second in a vertical position attached to the pulley system.

The syringe pump method was then tested with various volumes of bead dilutions and found to result in no lattice formation due to the beads having too sparse of a distribution. A third system was also proposed involving a microscope stage as the actuator mechanism. One problem with using the microscope stage is that it is ultimately hand operated, meaning there is no accurate control over the velocity. Another problem with the microscope stage is not being able to get the glass to fully pass through the liquid-liquid interface smoothly due to the small vertical displacement of the microscope stage. Therefore the syringe pump system was chosen over the microscope stage method.

Surface treatments

The glass microscope slides were also treated under different conditions and observed for any type of lattice-like structures. The glass treatments included: plasma treatment, APTMS (Aldrich®, Lot: BCBF4819V), and ethanol cleaned or untreated glass a control group. The plasma treated glass was thoroughly cleaned before plasma treatment and applied to the liquid-liquid interface by both the hand pulley system and syringe pump system. Both methods did not yield only random aggregations of beads similar to the untreated glass. The APTMS treated glass had a higher concentration of beads due to a higher charge difference between the glass surface and the bead charge however, under fluorescence microscopy only random aggregations of beads were observed.

Different bead dilutions from 5% to undiluted were also tested. The 5% bead dilution in ethanol yielded a bead distribution that was too sparse for a lattice like structure to occur. The undiluted solution of fluorescent beads yielded a random aggregation of high concentrations of beads. Changing the dilution of the beads did have an effect on the structures viewed under microscopy but no lattice like structures were observed.

Equilibrium time

Finally the equilibrium time of the beads was tested from 0 to 15 minutes. The equilibrium time refers to how long the beads take to reach the liquid-liquid interface in a monolayer after being injected. The beads were shown to have an equilibrium time of around 10 minutes but there were still diffuse amounts of beads present in the water layer of the interface. At 15 minutes the diffuse amount of beads did not change significantly and were still viewable. The equilibrium time did not seem to have an effect on lattice formation in our experiment.

Conclusion:

Using the method presented by Isa *et al (2010),* with significant modifications, has not yielded any promising results for our real time CTF method. The modification of injection rate, bead dilution, pulling rate, and glass treatment combined were unable to yield any lattice like bead structures. Though the bead lattices would be useful for calculating more accurate CTF results our method does not deem it a necessity to do so since traditional methods have proved to be easier while still yielding accurate results.

4.4.6 Micro-patterning grid

Micropatterning grid is a key component of our “grid + location indicator” design. Because the thickness of the original photo-etched coverslips exceeds the working distance of a

typical high magnification microscope objective, we decide to stamp the pattern onto the bottom of a regular coverslip.

Method:

Fabrication of PDMS stamp

1. Clean the photo-etched grid coverslips with ethanol and treat with oxygen plasma for 1 minute.
2. Mixing PDMS base and curing agent (Sylgrad®, Lot: 0006716335) at 10 :1 ratio and degas.
3. Glue the photo-etched coverslips (Electron Microscopy Sciences, Cat#: 72264-23) onto bottom of 35mm petri dish, with the grid side facing up.
4. Pour PDMS mixture into the petri dish
5. Cure in oven at 70^{o}C for 40 minutes.
6. Peer off the stamp from the glass.
7. Cure the stamp for an extra 30 minutes.

Preparation for stamping

8. Treat the surface of the stamp with oxygen plasma for 1 minutes.
9. Blow dry glutaraldehyde (Amresco®, Lot: 2842C052) treated glass.

Stamping

10. Coat the stamp with ink using Sharpie permanent marker.
11. Quickly put the glass onto the stamp and press hard for 20 seconds.
12. Peer off the glass with the stamp.
13. Use ethanol to clean the ink left on the stamp and the stamp can be re-used.
14. Bake the patterned glass in the oven at 100 ^{o}C for 40 minutes.

Results:

PDMS stamp: The PDMS stamp was prepared following the protocol described above has very clear extrusion of the pattern when looked under the microscope. After curing for extensive time, the pattern on the stamp is stable and does not wear off by shear force. However,

we've noticed that after 20 times of stamping, combined with the effects from ethanol wash, and plasma treatment, the extrusion of the pattern becomes shallower, which reduces the quality of the pattern on the glass as well. To address this problem, we can try other methods to further stabilize the PDMS stamp. Also, if we can produce the stamp more efficiently, then the time of use for each stamp is no longer a concern. Pattern on functionalized glass

In general, we manage to transfer the pattern onto the glass, but the consistency is an issue. For the successfully transferred patterns, the grid and the letters inside can be clearly recognized under microscope. Glutaraldehyde treated glass showed no difference in the quality of pattern transfer compared to untreated glass. However, the success of pattern transfer is all dependent on manual procedures, which are highly operator-dependent. In order to increase the reproducibility of our method, we are going to systematically quantify every step.

4.4.7 Image Stitching

Grid images are then produced by phase contrast microscopy. Figure 4-11 displays the grid images produced before stitching. The images must have overlap for proper image stitching. The images at each position are then stitched together using pairwise stitching in ImageJ. The result is a column of grid images as shown in Figure 4-12. The columns are then stitched together to produce a final grid image. The location indicator beads are then imaged which allow for cells to be found on the grid (Figure 4-13). The green location indicator beads are put through a different process of hand stitching through MosaicJ, a plugin in ImageJ, since pairwise stitching does not yield a correct final image. Once stitched a column is again generated (Figure 4-14). Multiple columns are then hand stitched in MosaicJ and cropped to generate a final green bead location indicator image which is critical for the final red bead image generation

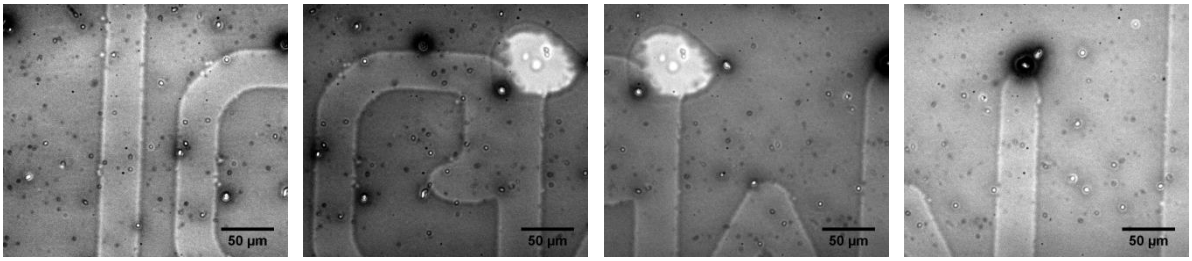


Figure 4-11: Grid Tiles

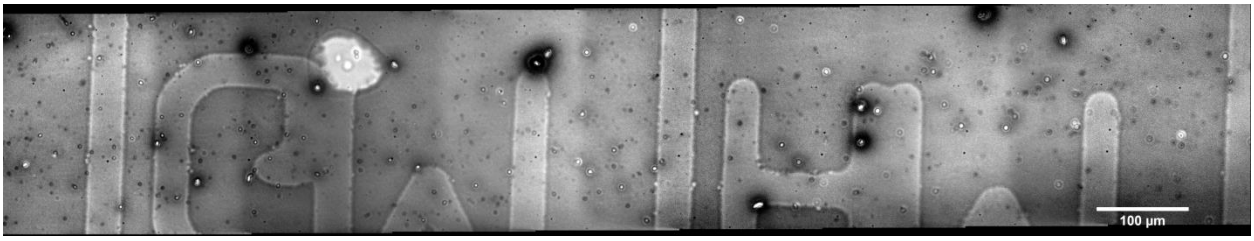


Figure 4-12: Grid Column Stitched

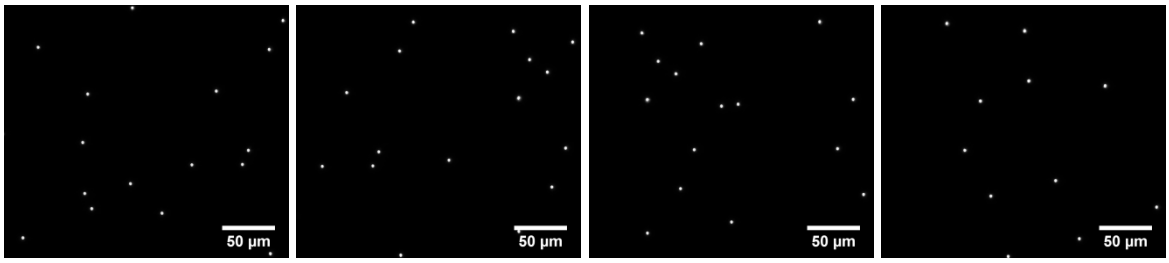


Figure 4-13: Green Bead Tiles

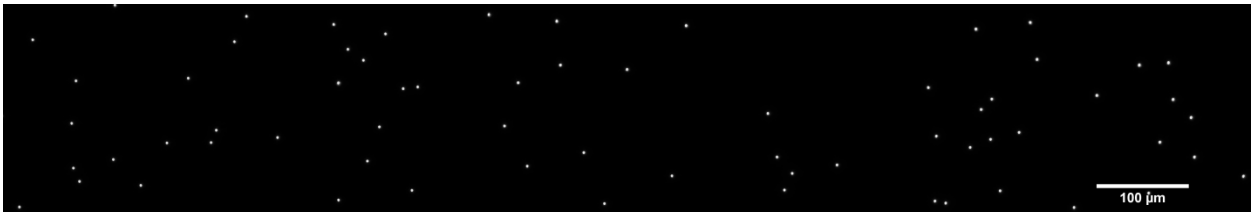


Figure 4-14: Green Bead Tiles Stitched

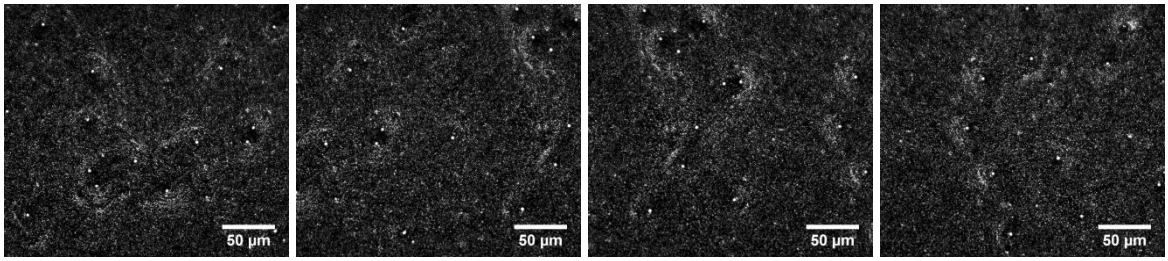


Figure 4-15: Red Bead Tiles

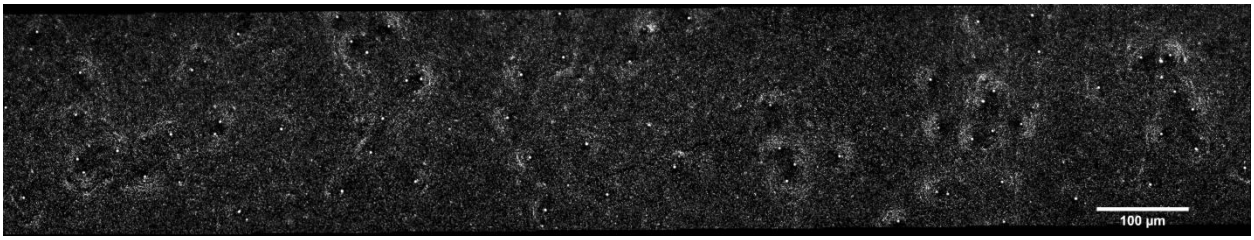


Figure 4-16: Red Bead Tiles Stitched

Finally red bead tiles are imaged under fluorescent microscopy (Figure 4-15). The position already predetermined generates specific tiles for the area from the grid area previously shown. The tiles must also be stitched.

The red bead tiles are stitched through the pairwise algorithm once again to generate a column (Figure 4-16). The columns are then stitched together to create the final red bead image needed to calculate CTFs. All three stitched images are vital for generating a final CTF measurement and for imaging multiple cells at once. In summary, the green beads and microgrid act as a location indicator system while the red beads are used to track hydrogel deformation ultimately leading to CTF measurements.

Chapter 5: Final Design

Summary of Methods for the Final Design:

5.1 Stamping of Micro-grid

A glass cover slide with a laser-etched alphanumerical micro-grid (square size- 500µm x 500µm, 20 x 20 squares) (Electron Microscopy Sciences, Cat#: 72264-23) was used as a mold to fabricate PDMS stamps. The grid was then stamped onto the bottom of a functionalized glass slide with permanent marker ink. The stamped glass slide was baked at 150°C for 1 hour. The stamping follows the protocol in the previous section.

5.2 Fabrication of PA Hydrogel

Red and green fluorescent beads (0.2 µm and 2 um size respectively) (Invitrogen^{TM,} Lot: 1010102 & 906909) were deposited on an oxygen-plasma treated glass cover slide at a mass ratio of 10:3. The green fluorescent beads were used as location indicators and red fluorescent beads were used for measuring gel deformation resulting from cell contraction. Polyacrylamide hydrogel (8% acrylamide 0.1% bisacrylamide, 7.5 kPa stiffness) was prepared using standard protocols. The acrylamide solution was sandwiched between the micro grid stamped glass slide and the bead-deposited glass slide. The sandwich allowed acrylamide polymerization and transfer of beads to the hydrogel simultaneously. The bead-deposited glass slide was then separated to expose the bead coated hydrogel surface. The hydrogel was then coated with protein to promote cell adhesion and exertion of traction force. The type of protein depends on the interested ligand. We have tested collagen (50 µg/mL), fibronectin (25 µg/mL) and laminin (100 µg/mL). These three coatings all provide favorable cell adhesion conditions. Figure 5-1 summarizes the workflow of the gel fabrication process. The detailed protocol for the gel fabrication procedure is included in Appendix 4.

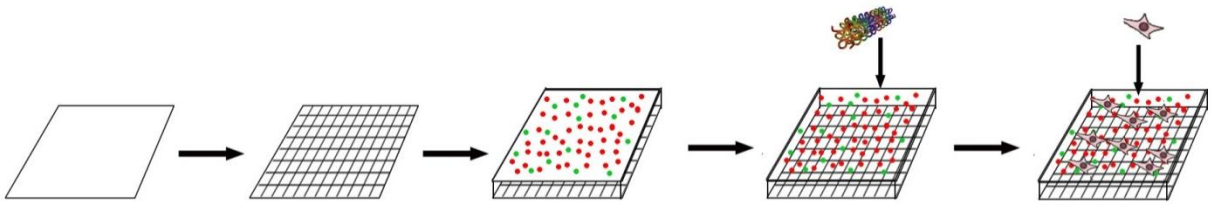


Figure 5-1: Workflow of the gel fabrication processes.

5.3 Image Analysis

Multiple overlapping microscopic images, covering a 1mm X 1mm gel area, were taken and stitched in ImageJ software. The positional shifts of the red fluorescent beads were measured using Particle Image Velocimetry (PIV) in MATLAB®, from which the gel surface deformation was mapped. The PIV analysis results were then imported into ANSYS® software for Finite Element Measurement (FEM).

5.4 Component Assembly

The final design to obtain real time cell traction force measurements involves the usage of: a six well plate base (CytoOne, Cat: CC7682-7506), a petri dish (BD Biosciences, REF: 351006), micro grid stamped on a glass slide, a deformable hydrogel layer on the slide, fluorescent beads placed in the hydrogel.

After the micro-grid has been stamped on to the glass it is then placed upside down (microgrid on the bottom surface) and glued to a petri dish with a hole in the center. The reason for flipping the grid is so the grid is not washed away or interfering with the hydrogel and cells. The next step involves assembling the polyacrylamide hydrogel on the micro grid glass.

Once the hydrogel and fluorescent beads are assembled cells can be seeded onto the substrate. The petri dish is then glued to a six-well plate which fixes the plate on the microscope stage free of rotation.

The cells are then imaged under phase contrast and fluorescence microscopy. The images produced from the procedure allow for image stitching and a larger area to be imaged. The figures below show how the red bead images, grid, and green beads can be used for identification of cells and calculation of cell traction forces.

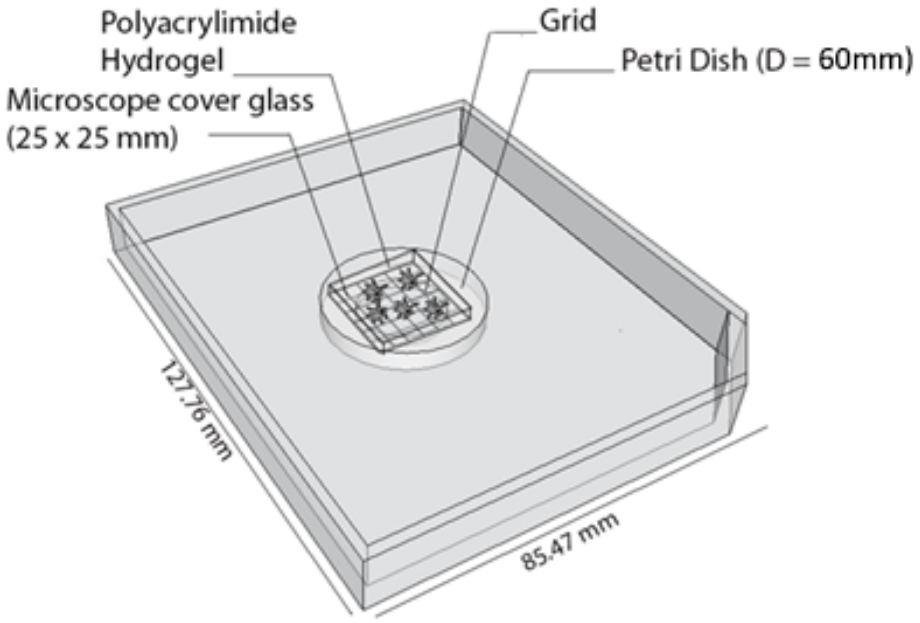


Figure 5-2: CAD Drawing of Real-Time CTF Device

In Figure 5-2 the dimensions of the device are shown. The base six well plate has a length 125.76 mm and a width of 85.47 mm. The base plate has a hole punched through the center with a petri dish glued on. The petri dish also has a hole punched through the center with a glass cover slide glued onto the bottom. The petri dish has a diameter of 60mm and the glass cover slide has

dimensions of 25 mm by 25 mm. The hydrogel, fluorescent beads, and cells are all placed on top of the glass slide after the micro grid has been stamped to the bottom side.

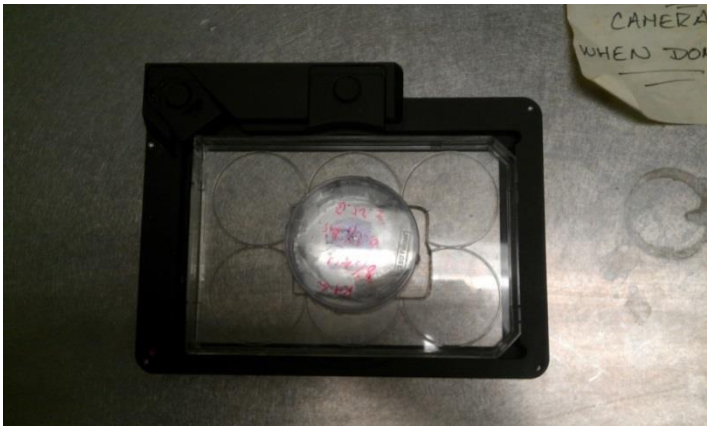


Figure 5-3: Device placed in microscope stage holder

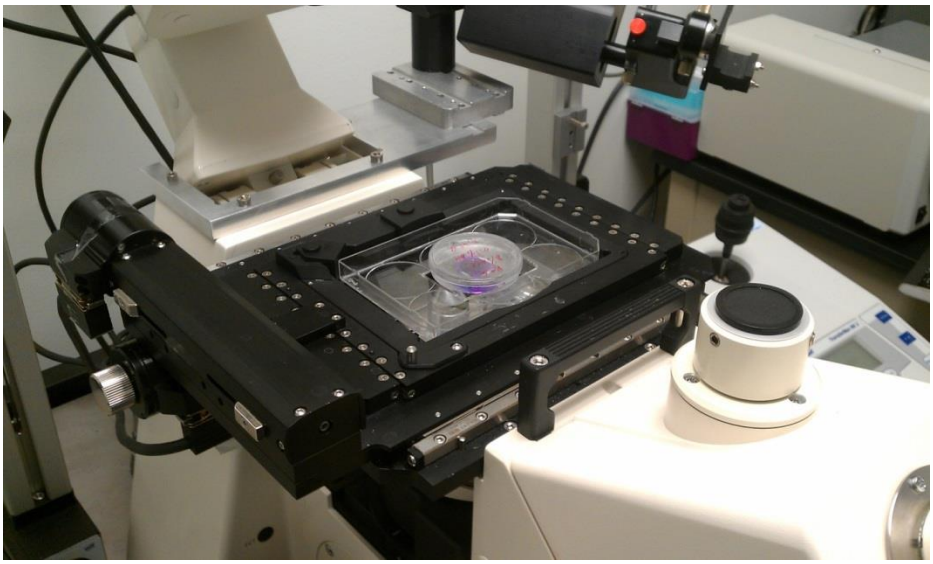


Figure 5-4: Device placed onto microscope stage

The device is then placed into a holder used for six well plates. The holder keeps the device stable and eliminates rotation while imaging is taking place. Figure 5-3 displays the device secured in the holder with cells, beads, and hydrogel.

Once the device is securely in place it can be mounted on the microscope stage. Before imaging, final adjustments can be applied as shown in Figure 5-4.

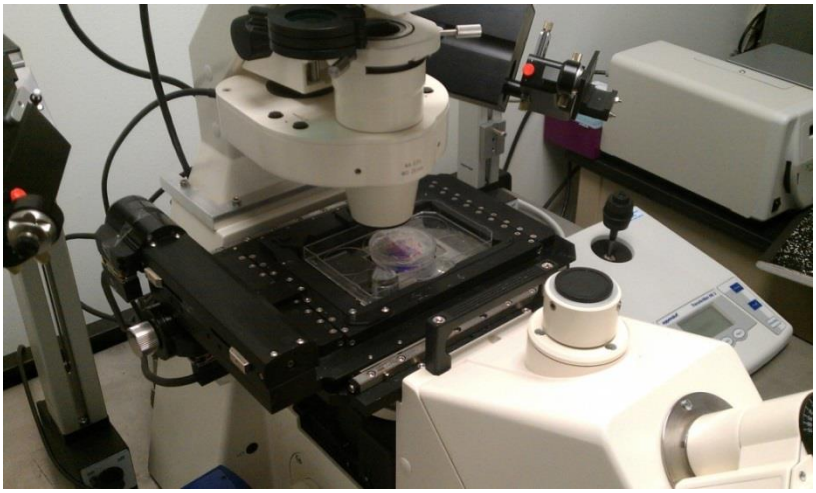


Figure 5-5: Device during microscopy

During microscopy the stage will move and take images at predetermined positions under phase and fluorescent filters. Figure 5-5 displays the importance of having a secure device because movement would cause unclear images to be produced and rotation would give inaccurate CTF measurements.

Chapter 6: Design Validation

6.1 Rotation testing:

In order to allow valid data for CTFM analysis, our design needs to minimize the possibility of rotation between multiple times of mounting the plate onto the microscope.

We mounted the plate onto microscope stage twice, and each time we measured the relative angle between the grid lines and horizontal direction in ImageJ software. The comparison between the first and second measurements is shown in the table.

Sample ID	Angle (Degrees)	Difference
7D10x	90.457	0.334
7D10x2	90.791	
7D20x	89.599	0.718
7D20x2	90.317	
DS10x	89.507	-1.106
DS10x2	88.401	
DS20x	91.244	-0.883
DS20x2	90.361	
GN10x	89.693	0.142
GN10x2	89.835	
GN20x	90.55	-0.779
GN20x2	89.771	
Absolute difference value Average (Degrees)		0.660

Table 6-1: Rotation Angle Results

The average is 0.66 degrees, which is negligible for PIV analysis. Also, the difference in the degrees has relatively large variations most likely due to human error during placement of the device on the stage. This indicates that the difference may not come from rotation of the device, but from errors in manual measurements. In short, this test showed that our design reliably minimized the possibility of rotation when mounted on the microscope stage.

6.2 Position identification

The next step in validating our design involved being able to stitch together red bead images at a high enough resolution to enable force vector calculations. The red fluorescent beads in a whole grid area are imaged by taking multiple high magnification fluorescence images, each

covering roughly a 200 by 200 µm area. The images were then stitched together using ImageJ. The resulting stitched bead image area can be seen in Figure 6-1. Upon closer inspection under a very high resolution the beads can clearly be seen meaning there is little to no blurring.

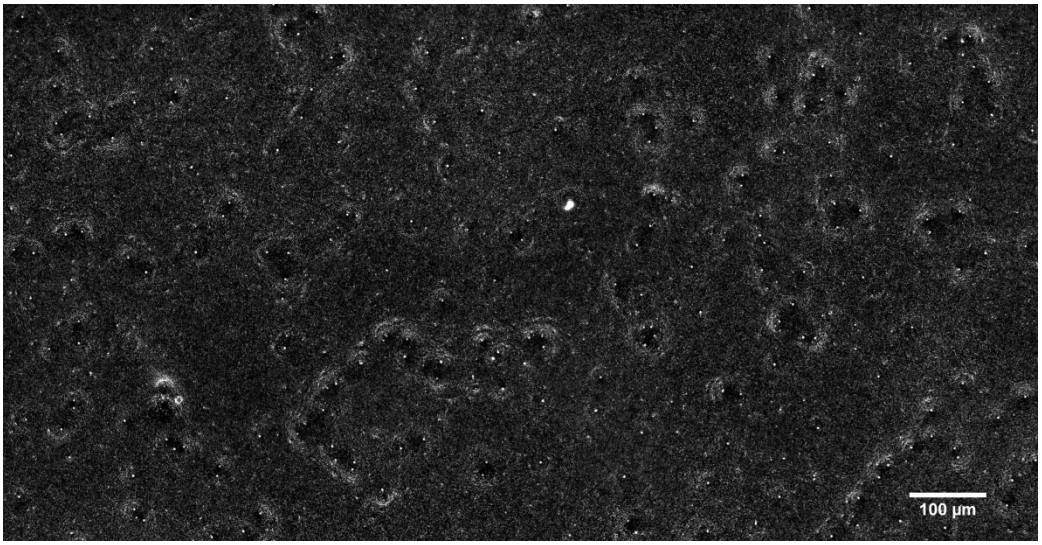


Figure 6-1: Stitched Red Bead Area

An image of the microgrid was also taken under phase contrast microscopy. The image validates the location indicating properties of our microgrid. Figure 6-3 shows on the cellular plane that there are a certain amount of cells with a specific configuration in each block of the microgrid. The image was taken under 10x objective to show both the grid and individual cells.

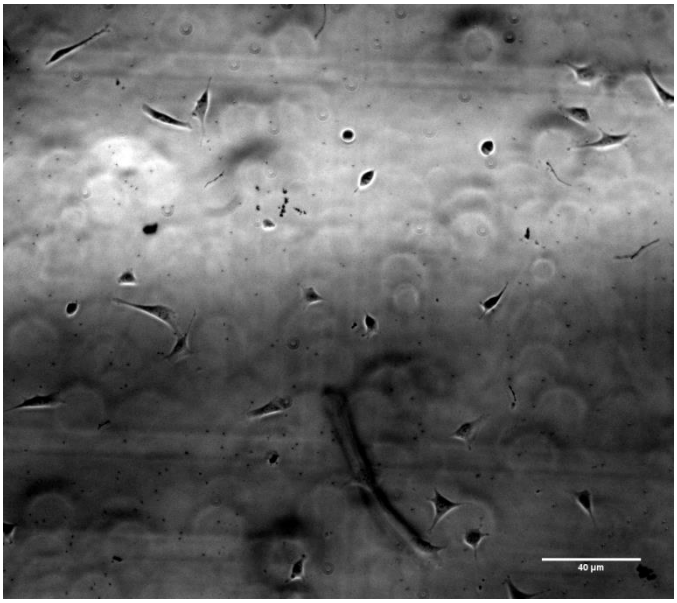


Figure 6-3: Cells and Microgrid (10x objective)

The next step involves stitching together the microgrid images. The microgrid was also imaged under 40x with phase contrast microscopy generating roughly 200 x 200 µm blocks. The blocks were then overlapped multiple times over two squares of the grids. Figure 6-4a shows the microgrid 40x block. Figure 6-4b is the result of stitching together multiple microgrid blocks to form two squares of the microgrid.

The stitched image is done before any cells are loaded onto the hydrogel. By stitching together the grid, red bead, and green beads into a specific area a before and after image comparison can be done. The comparison involves red bead displacement generating the force vector map which ultimately leads to CTF measurements. The next step explains generating the results in more detail.

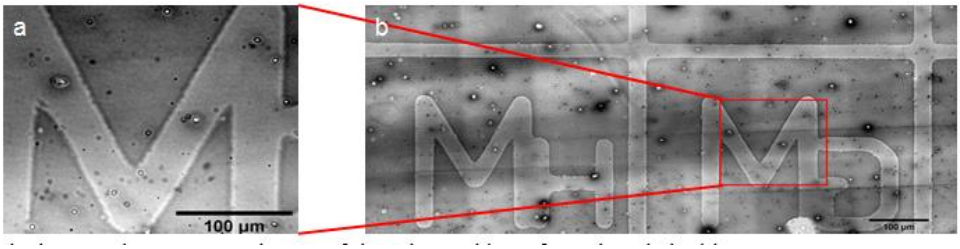


Figure 6-4: (a) Microgrid Block, (b) Microgrid Stitched

Once the hydrogel has been seeded with cells it can be considered loaded because the cell is applying a force or load. The hydrogel is then imaged under phase contrast microscopy (Figure 6-5a) generating a visible cell image and specific green bead pattern. The green bead pattern is then generated under fluorescent microscopy (Figure 6-5b) which represents the location of the cell when the gel is loaded. The loaded green bead pattern is then used to locate where the cell is placed on the hydrogel by comparing it with the initial stitched green bead pattern (Figure 6-5c).

Once the green bead pattern is located the corresponding red bed pattern can also be found and matched with the initial red bead configuration (Figure 6-1). The comparison of the two ultimately results in the final CTF measurements. Being able to locate the green bead pattern multiple times makes real time analysis achievable.

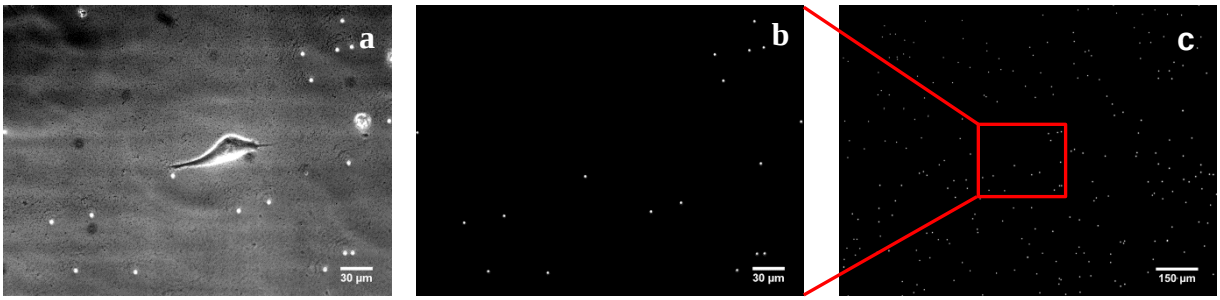


Figure 6-5: (a) Loaded Hydrogel Cell Phase Contrast Image, (b) Loaded Green Fluorescent Image, (c) Unloaded Stitched Green Fluorescent Image

6.3 Cell Traction Force Measurements

We tested our method by measuring cell traction force of NIH/3T3 fibroblast, without removing the cells from the hydrogel. Before seeding cells, the hydrogel surface was coated with 20 µg/mL fibronectin. The plate was then mounted onto the motorized stage of a Zeiss Axiovert 200M inverted microscope. We imaged a total area of 2 grids (~1mm x 0.5mm area) under 40x magnification. The size of each image was 260 um x 240 um, separated by a spacing on x and y direction of 150 um. This gives 110-90 um of over lapping. The total area includes 8 x 4= 32 images in each of the channels: Red fluorescent, Green fluorescent and Phase contrast (for the grid). The individual images were then stitched in Image J software.

NIH/3T3 fibroblasts were seeded onto the gel at ~10% confluence, and incubated overnight. The following morning, the plate was mounted onto microscope and we found the previous imaged location by the letter coordinates. Within the imaged area, 5 cells with sufficient separation to other cells and healthy morphology were selected. The cells were imaged using a 40 x objective, in all three channels (red, green, phase).

One cell was selected to validate that CTF measurements could be taken under normal conditions. The cell was initially imaged under phase contrast to generate a green bead configuration as stated before (Figure 6-5a). After the green bead configuration was found the cells red bead configuration was found manually and cropped from the initial stitched image.

After being cropped from the stitched image two images of the red bead configuration (before and after contraction) were loaded to MATLAB® A map of gel deformation (Figure 5b) was generated by analyzing the bead displacements using MPIV, a PIV toolbox in MATLAB

(Mori and Chang, 2003). The deformation map displays where the cell is contracting and in which direction.

Finally the gel deformation data was loaded to the ANSYS® for Finite Element Analysis (FEA) to calculate the traction stress applied on the gel surface by the cell. The FEA stress data was then reloaded to MATLAB® and a map of the stress with overlaying the cell outline was generated (Figure. 6-6c). The stress map shows that the cell has its highest contraction at the red areas (~140 Pa) and the corresponding force vector matches the stress map. The results validate that our method works under normal control conditions.

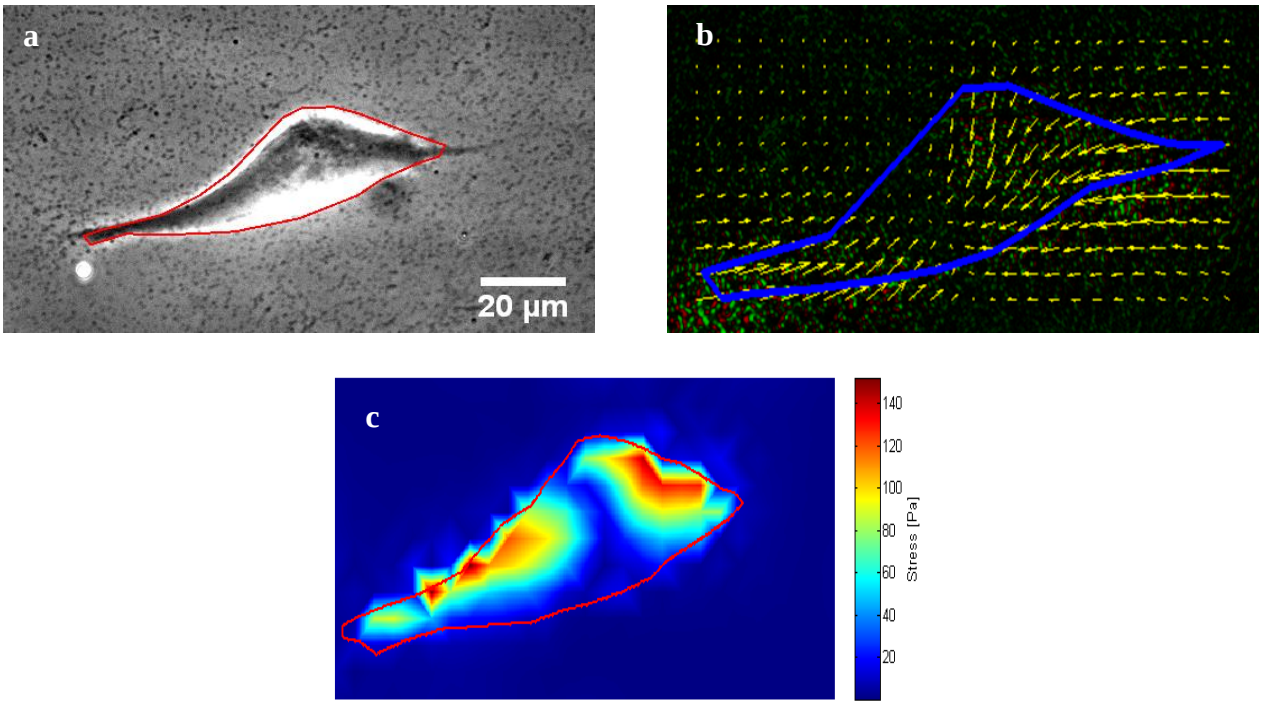


Figure 6-6: (a) Phase Contrast Cell Image (40x), (b) Deformation Vector Map, (c) Stress Map

In summary, we have shown that our method allows for cell traction force analysis in real time without removing/ damaging the cells from the substrate. The next step is to measure cell traction force multiple times over the course of ~1-2days, to monitor the change of cell traction force in different time frame.

6.4 Real-time monitoring of CTF

The method was tested to measure the CTFs of NIH/3T3 fibroblasts on fibronectin coated hydrogel multiple times over 2 days. The experiment protocol is summarized below:

1. An image of a 1mm x 1mm area was acquired by stitching multiple images taken under 40x objective prior to cell seeding.
2. NIH/3T3 fibroblast cells were seeded and incubated overnight.
3. Selected cells within the area were imaged. The corresponding position of the cell in the original image was later identified by recognizing green bead configuration within the grid square.
4. Gel deformation due to contraction was measured by determining positional shift of red fluorescent bead configuration between the two time points.
5. Image analysis was done using PIV and FEM.
6. After each measurement, the cultures were returned to the incubator for imaging at later time points.

Using the reference bead configuration we were able to locate a singular cell 42 hours after seeding under phase microscopy (Fig. 6-7a). Once the cell was located a force vector map was generated once again (Fig. 6-7b). A stress map for the cell was also generated with the highest stress being at the edges of the cell (Fig. 6-7c).

Finally, we were able to locate the same cell 3 hours after initial imaging (Fig. 6-7d). A separate force vector (Fig. 6-7e) and stress map (Fig. 6-7f) were also generated. The morphology of the cell was changed (Fig. 6-7a) due to the dynamic nature of cell migration when compared to its initial configuration (Fig. 6-7d).

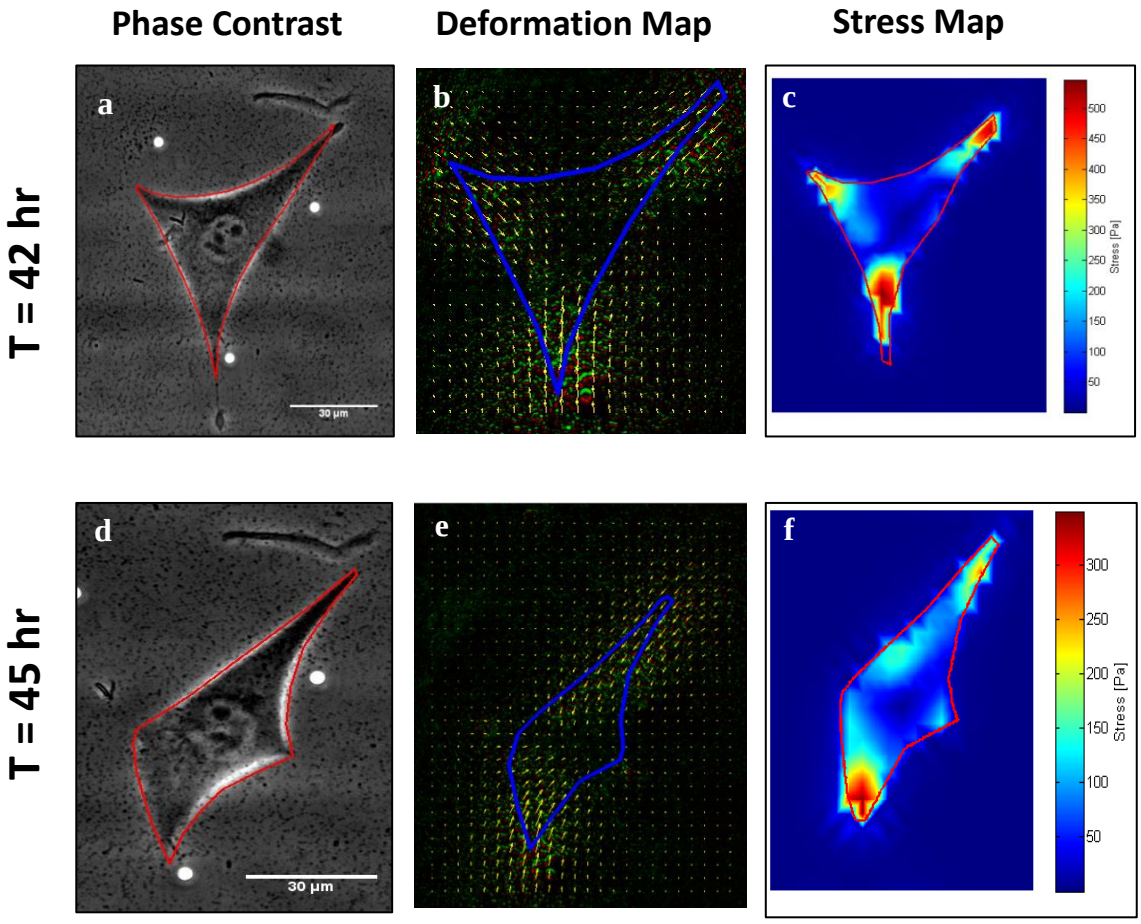


Figure 6-7: (a) Phase Contrast Cell 42 Hours After Seeding (40x), (b) Deformation Vector Map, (c) Stress Map, (d) Phase Contrast Cell 45 Hours After Seeding (40x), (e) Deformation Vector Map, (f) Stress Map

By locating the same cell over a period of 3 hours and generating CTF measurements our goal of real-time analysis was fulfilled. The morphology of the cell and the force of the same cell

was also changed (7.72×10^{-8} Newtons to 6.39×10^{-8} Newtons). The bead configuration and location indicator system was validated through these results.

Chapter 7: Discussion

Our design validation results demonstrated that our method succeeded in measuring cell traction forces of NIH/3T3 fibroblasts multiple times over days, without removing the cells. The calculated values for CTFs were within the typical range for NIH/3T3 fibroblasts on 7.5 kPa substrate with fibronectin coating (20-200 nN).

Being able to measure CTFs in real time has many potential applications in mechanobiology research. Monitoring the CTFs of a single cell allows researchers to study the change of cell contraction at different stages in the cell cycle or during different cellular processes. Previously, single-cell traction forces were measured as a static parameter. However, the dynamic mechanism of cellular behaviors in different stages in the cell cycle needs to be considered. The limitation with traditional CTFM is that it requires the cells to be removed to generate one measurement, which prohibits long-term monitoring of the change in CTF.

An alternative method to measure CTFs of a single cell in multiple time points is to image the fluorescent bead configuration at one location multiple times and eventually remove the cell to provide the unloaded comparison. One example of such studies was reported by Munevar and co-workers, (Munevar *et al*, 2001). They monitored the CTFs of migrating H-ras transformed NIH/3T3 fibroblasts over 30 minutes. Imaging the bead configuration at a fixed position significantly limited the time period allowed for the monitoring, as the cell might

migrate out from the viewing area. However, our method overcomes this limitation by imaging and stitching a large area of the gel surface.

Our method also offers great potential in testing the effects of specific chemicals, drugs or genetic modifications on cell traction forces. Previously, studies on the effects on CTFs brought by specific treatments were done only on a statistical basis. Because the cell has to be removed for one measurement to be taken, the same cell's condition before and after the treatment cannot be directly compared. For example, Luna and co-workesr, conducted a study on the effects of miR-200c transfection in trabecular meshwork cell contraction (Luna, *et al*, 2012). In this paper, the authors interpreted the data on a statistical basis by comparing the average of the control and transfected groups, and used t-test to demonstrate significant difference. In contrast, our methods allow CTF measurements to be taken without removing the cell, which means the cells can be manipulated with the treatment of interest between measurements. After the treatment takes effects, the same cells can be found with our position indicator system and measured again. The direct comparison allowed by our method would certainly carry more weight in demonstrating the treatment's effects.

In addition to our original goal to allow real-time measurement, our method also allows measuring of CTFs in a cluster of cells. Studying CTFs of a cell cluster or aggregate is important in understanding the mechanical cell-cell interactions. Traditionally, the area of the cluster is limited by the viewing area of a microscope under high magnification. Because our method involves stitching individual images to form a large image, we are able to overcome this limitation and enable the simultaneous CTF measurement of large clusters of cells (Figure 7-1).

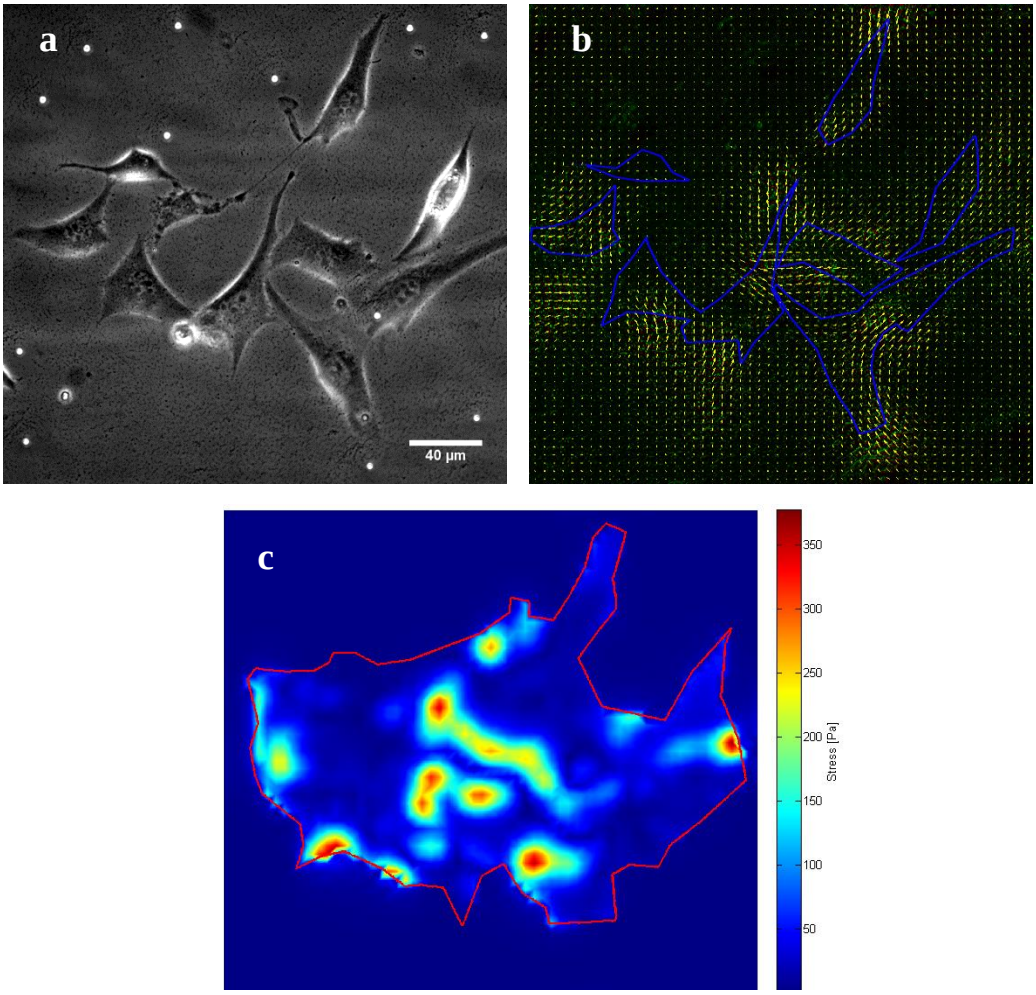


Figure 7-1: (a) Phase Contrast Cluster of Cells (40x), (b) Deformation Vector Map, (c) Stress Map

7.1 Economic Influence

Our design primarily serves to facilitate research in Cellular Biomechanics. In terms of economic influence, because our method will not be implemented directly in the industry, the significance in economy is minimal. However, our method does provide a cost-effective way for conducting relevant research. All the materials we use are easily obtainable and low-cost. In particular, by allowing the sample to be transferred back in the incubator between measurements, our method detours the need for a microscope environment chamber, which typically costs over $10,000.

7.2 Environmental Effects

The method we develop is an in vitro technique to measure CTF. Because the design is implemented at cellular scale, the wastes produced are in very small quantity. There is no concern on the sustainability and environmental effects.

7.3 Health and Safety Concerns

The use of the product is restricted in research labs with standard safety regulations. It will not be distributed to the general public. Health and safety is a minor concern, because all procedures involved should be conducted in a Bio-safety Level- 2 laboratory setting according to the regulations. Different cell types may be tested, and the specific safety instructions are dependent on the biohazard related to the samples of interest.

Furthermore, social, political and ethical concerns aren't significantly relevant to our project. Our product does not include controversial components like stem cells, neither does our product involve in animal/ human subject testing. It only provides an in vitro model for studying the mechanics of cells explanted from living systems.

7.4 Manufacturability

In terms of manufacturability, all components in the design are currently fabricated and assembled manually. The manufacturing does not require any complicated procedures and can be conducted with common tools available in most research laboratories. However, for future development, we do recommend automated manufacturing of the device, in order to improve the efficiency and reproducibility.

Chapter 8 Conclusion and Future Recommendations

In conclusion our real time cell traction force method fulfilled our goals and objectives set forth in our revised client statement. We were able to take multiple CTF measurements over a period of time without removing cells from our polyacrylamide substrate. The results of measurements are consistent with published traction force values for 3T3 fibroblasts.

The combination of the location indicator green fluorescent beads and the microgrid allowed for identification of multiple cells between each reincubation period. Once identified we were able to generate accurate CTF results. On top of being able to identify singular cells we also used our method on clusters of cells. Through the use of image stitching clusters of cells can also be analyzed for their CTFs. Another opportunity for generating new data could involve seeing how these cells CTFs interact at their contact points.

Though our method has succeeded in fulfilling our objectives there are also many recommendations that should be taken into account for the future.

Currently multiple 200 x 200 µm images are acquired from the microscope and then stitched together manually using ImageJ to give an image of a large sampling area. Depending on size of the area, this process can be very time consuming. If the microscope software can be programmed to automatically image and stich a defined area of the gel, the process can be done much faster over larger areas, allowing for more data generation.

The manufacturing process of the device includes: gluing the petri dish to the six well plate, and punching holes through both the six well plate and petri dish. One problem with each process is that gluing manually can result in an uneven surface causing blurry images. Punching

holes through each dish manually also has similar results. If the process was machine automated then a better device with higher tolerances could be obtained allowing for more reproducible results.

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Appendix 1 - Pairwise Comparison Chart

Revised Pairwise Comparison Chart

	Relatively high resolution	Mimics *in vivo* mechanical environment	Sensitive measurements	Multiple measurements	Reproducible	Precise	Easy fabrication through protocol	Easy to handle	Total
Relatively high resolution	X	0	0	0	0	0	0,5	1	1.5
Mimics *in vivo* mechanical environment	1	X	1	0.5	1	1	1	1	6.5
Sensitive measurements	1	0	X	0	0.5	0.5	1	1	4
Multiple measurements	1	0.5	1	X	1	1	1	1	6.5
Reproducible	1	0	1	0	X	1	1	1	5
Precise	1	0	0.5	0	0	X	0	0	1.5
Easy fabrication through protocol	0.5	0	0	0	0	0	X	1	1.5
Easy to handle	0	0	0	0	0	0	0	X	0

Appendix 2- Function Means Chart

Functions	Means			
	Self Assembling Bead Lattice	Reference Bead Method	Bead Projection Method	Micropillar Method
Embed beads on top surface of hydrogel	Preformed lattice of beads embedded on hydrogel	Two types of fluorescent beads are randomly distributed	Beads randomly distributed	Beads are stamped on hydrogel
Allow measurements of CTF under microscope	Tracing displacement of lattice	Tracing displacement of beads	Tracing displacement of beads	Tracing of displacement of beads
Enable real time measurements	Initial lattice structure compared directly to loaded image	Uses reference beads to identify location on the gel for comparison with initial image	Comparison of bead distribution on coating layer	Initial lattice structure compared directly to loaded image

Appendix 3- Evaluation Matrix

Design Constraints (C) and Objectives (O)	Self Assembling Bead Lattice	Reference Bead Method	Bead Projection Method	Micropillar Method
C: Should not damage cells				
C: Fluorescent beads should be visible in microscope			?	
C: Budget				?
O: User Friendly	90	60	90	90
O: Multiple measurements	90	90	90	90
O: Reproducible	40	90	20	50
O: Sensitive Measurements	90	80	80	90
Total	310	320	280	320

Appendix 4 - PA Gel Fabrication Protocol

A) Preparing intermediate glass deposited with beads:

1) Put slide cover glass (Corning, Cat#: 2865-25) in ethanol (Fisher Scientific, Lot: 112499) and sonicate for 5 minutes.
2) Blow dry the glass.
3) Treat the glass with oxygen plasma for 1 minute.
4) Suspend red fluorescent beads (0.2 µm diameter) (Invitrogen^{TM} , Lot: 1010102) at 0.5% concentration, and green fluorescent beads (2 µm diameter) (Invitrogen^{TM,} Lot:906909) at 0.15 % concentration in ethanol
5) Sonicate the bead suspension for 5 minutes
6) Place the plasma treated glass on heat cube and drop 50 uL of bead suspension on each glass. Try to let the liquid spread uniformly on the glass.
7) Move the heat cube and glass into vacuum oven at 150 °C and 20 mmHg vacuum pressure to allow the evaporation of ethanol.
8) Take out the glass from oven after 30 seconds. Fluorescent beads should be uniformly left on the intermediate glass.

B) Functionalization of the bottom glass:

1) Put slide cover glass in ethanol and sonicate for 5 minutes.
2) Blow dry the glass.
3) Merge the glass in 1% APTMS (Aldrich®, Lot: BCBF4819V)
4) , put on the stirrer for 30 minutes.
5) Take the glass out from the APTMS solution, rinse with DI water 3 times.
6) Put the glass in oven to dry the excessive liquid.
7) Put the glass in 0.5% glutaraldehyde solution (Amresco®, Lot: 2842C052) in fridge for at least 3 hours.

C) Polyacrylamide gel fabrication

1) Prepare the acrylamide/ bis solution. (Bio-Rad Cat# 161-0140, 161-0142)
2) Allocate the solution in 250 uL per tube.
3) Thaw 10% Ammonium Persilfate (APS) solution (Amresco®, Lot: 1301C035). Once thawed, keep the APS in ice.

4) Take the functionalized bottom glass from the glutaraldehyde solution and dry with vacuum.
5) For the 250 uL tube, add in 2.5 uL of 10% APS and 0.9 uL of TEMED (Amresco®, Lot: 2341C079) to initiate polymerization
6) Quickly drop 64 uL of gel solution onto each bottom glass and cover the solution with the intermediate bead glass.
7) Wait for 7 minutes to allow polymerization of the gel.
8) Carefully peel off the intermediate glass, the gel should remain on the functionalized bottom glass with fluorescent beads embedded on the top surface.
9) Keep the gel hydrated with HEPES or PBS buffer.