Developing a Three-Dimensional Microenvironment to Investigate Metastatic Cancer Invasion

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Abstract

Significant progress has been achieved towards understanding the biochemical aspects of cancer metastasis; yet, less is known about the mechanical processes that govern cancer cell invasion. These mechanical processes include: 1) changes in the elastic properties of cells and 2) changes in the motility of cells triggered by physical properties of the microenvironment. This thesis aims to understand the latter; in particular is the traction behavior of cancer cells as they navigate through different pore sizes. Cells were seeded on 3D hydrogel scaffolds with a structure and dimensionality that mimic the physiological conditions encountered by metastatic cells \textit{in vivo}. Migratory behavior of these cells were determined by traction force microscopy, a technique used to determine the forces generated by cells on their substrata. Data on cell tractions allowed analysis on the mechanical interplay between cells and their environments. Understanding this interplay can have profound implications on cancer therapy, such as reducing or preventing the migration of these cells.
1.1 Mechanics in Cancer Metastasis

While biochemical aspects of cancer cells, such as gene regulation and expression, have been studied in detail, much less is known about the mechanical processes (e.g. cell movement and motility) that govern cancer cell invasion. Recently however, cancer biologists have begun to appreciate the latter as they recognize that an important part of metastasis involves changes in the physical properties of the cell and the extracellular matrix [1,2]. As the name suggests, the extracellular matrix is the “extracellular,” or exterior part of tissue that fills the spaces in between cells. This matrix, which holds tissue together, is composed of macromolecules including fibers and polysaccharide chains. Examples of these fibers are collagen and elastin, which provide strength and flexibility. Polysaccharide chains, made up of at last three sugar molecules, hydrate the matrix and permit the rapid diffusion of nutrients [3]. Although the primary role of the extracellular matrix is to provide physical support for tissue, it has been shown to regulate cellular behavior. For example, reducing the matrix stiffness has been shown to impede cell spreading and migration [4,5]. Additionally, benign cells can be transformed into malignant ones by simply changing the stiffness of the matrix to closely resemble that of tumor tissue [1]. These observations illustrate the critical role of cell-matrix interaction in regulating cellular properties such as tumor metastasis, wound healing, and angiogenesis (the formation of new blood vessels) [6].

Cancer cells encounter a number of mechanical forces as they transition into malignancy (Figure 1). These forces include forces from neighboring cells or the extracellular matrix and shear forces from blood flow [1]. Cells continuously experience mechanical forces from their
environment and in response, exert reciprocal forces. To better understand these cell-matrix interactions, cellular forces and motions must be measured and analyzed [7]. Many techniques for quantifying cell tractions have been developed in the last few decades, but traction force microscopy remains the most commonly used. In this method, fluorescent particles embedded in a substrate gel are tracked using a confocal microscope [8,9]. This microscope gathers light in a pinhole and eliminates out-of-focus light, enhancing optical resolution and allowing imaging in the depth direction. Displacements of these beads are then used to determine cell tractions based on the theory of elasticity [6]. A brief discussion of this theory is found under the “Results and Discussion” section in Chapter 3.

Figure 1. Mechanical forces experienced by a tumor cell. As a cell transitions into malignancy, it experiences mechanical forces from its environment. Even in normal tissues (A), cells constantly encounter a variety of forces such as forces from their neighbors. As the cell begins to detach from its primary tissue (B), it generates tractional forces during locomotion. Once the cell reaches the vasculature (C), it experiences shear forces from blood flow. Finally, for a cell to metastasize (D), it must migrate through intact walls of blood vessels. Their migration introduces additional mechanical forces to their surroundings. The image was taken from Kumar and Weaver [1].
1.2 Previous Works

Recent works have yielded valuable insight into the mechanical properties (e.g. cell stiffness and cell tractions) of cancer cells. Understanding how these properties differ between cancer cells and normal cells can have profound clinical implications. For example: instead of surgically removing tissue and observing it under a microscope for signs of disease, pathologists can detect cancer cells based on cell stiffness, matrix stiffness, and cell tractions. Cancer diagnosis based on these properties can be potentially more accurate than routine morphological analysis since cancer cells often resemble the healthy cells of the organ from which they originate [10]. Below is a discussion of three studies that focus on the mechanical, rather than the biochemical, aspect of cancer cells. Their results suggest that changes in the physical properties of the cells and measurement of cell tractions have potential in distinguishing cancer cells from normal cells.

Cell Stiffness

In a study by Cross et al., the stiffness of cancer cells taken from patients with lung, breast, and pancreas cancer was characterized by atomic force microscopy [11]. The stiffness of cancer cells was found to be 70% softer than benign cells with no overlap in the observed values. The change in stiffness is hypothesized to aid in facilitating easy migration during metastasis [12]. Cancer and benign cells were also found to display different trends; elasticity measurements for cancer cells were best fit by a Gaussian curve while that for benign cells were best fit by a log-normal fit. In other words, when the number of cells was graphed as a function
of elasticity, the curves for cancer and benign cells resembled different mathematical functions. The curve for cancer cells can be fitted with the equation:

\[ y = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(x-\mu)^2}{2\sigma^2}} \]

while the curve for benign cells can be fitted with the equation:

\[ y = \frac{1}{\sigma\sqrt{2\pi}} e^{-\frac{(ln(x) - \mu)^2}{2\sigma^2}} \]

The fact that the two trends can be fitted by different curves provides a novel method for differentiating healthy cells from cancerous ones. These results were true for lung, breast, and pancreas cancer, indicating that the same mechanical analysis can be applied to different cancer types. While cancer is biochemically diverse, this study demonstrates that mechanically, a common modulus is shared between different tumor types.

Cell Traction

Li et al. studied the contractile forces of normal, benign, and malignant cells [13]. Cells were cultured on silicon nanowire arrays and any deflections (bending) of the nanowires were used to derive cell tractions. Bending of the nanowires could be seen through a scanning electron microscope and the forces required to induce bending was quantified by atomic force microscopy following a procedure previously described [14]. It was found that benign and malignant cells generated larger cell tractions than normal cells, a result confirmed by Kraning-Rush et al [15]. The large generation of force was attributed to the increased contractile forces necessary for cells to escape and metastasize. Both studies suggest that the larger tractions exerted by cells of metastatic potential may act as a mechanical marker for metastasis.
Integrin Expression

Cell migration during metastasis requires integrins, adhesion molecules that mediate attachment of a cell to its environment. A study done by Mierke et al. demonstrated a correlation between expression of α5β1 integrin and tumor cell invasion [16]. It was found that high α5 integrin expression correlated with increased invasiveness whereas a knockdown of α5 integrin decreased invasiveness. Invasiveness was quantified by a score defined as: cell number density per 1 mm² multiplied by the average invasion depth. A score below 0.1 mm⁻¹ was defined as non-invasive and a score above 0.1 mm⁻¹ was defined as invasive. To determine whether the difference in invasiveness was due to contractile forces, researchers measured cell tractions and found that α5β1high cells had tractions 3.5-fold higher than that of α5β1low cells. These data indicate that α5β1-integrin-mediated increased invasiveness is due to increased generation of contractile force. If so, then blocking the integrin with anti-α5 or anti-β1 integrin antibodies will lead to a dramatic decrease in invasiveness.

1.3 Motivation for Thesis

Many studies that have attempted to discover biological markers for metastasis have experienced marginal success. Since metastasis is foremost a mechanical process, changes in the physical properties of cells may serve as better indicators of cancer. One such property is the migratory behavior of cancer cells as they navigate through the extracellular matrix. Before cancer cells are able to spread and metastasize, they must first come in contact with a substrate and build physiochemical linkage for focal adhesion [17]. Once the cells complete this mechanical signal transmission, they begin to generate internal tensile forces required for
migration. This force, which is transmitted to the extracellular matrix, is called “cell traction.” Since cell traction is essential for cell migration, a complete knowledge of it is critical in understanding pathological events at the cellular level.

To study cellular behavior, we must first construct a model as physically relevant to physiological conditions as possible. Most studies done on cell migration have been carried out on two-dimensional substrates [18]. For example, asymmetric patterns on 2D substrates have shown to direct cell motion [19, 20]. Cells have also been shown to move persistently in one direction in straight microchannels used to simulate confined paths in tissues [21]. The physiological environment however, is diverse and non-uniform, and studies have shown that 2D substrates do not adequately mimic the environment of 3D native tissue [22]. To better depict the environment of native tissue, a 3D model is needed. In 2005, it was shown that 3D hydrogel scaffolds with inverted-colloidal-crystal geometry could support cell culture [22, 23]. From there, studies have shown that these scaffolds could also be used to study cell behavior such as adhesion and migration. Together, the hydrogel and inverted-colloidal-crystal geometry have the benefits of: 1) high porosity for the penetration of media, 2) interconnected pore network for cell migration, nutrient transport, and waste removal, 3) appropriate chemistry for cells to attach to and proliferate in, and 4) ordered structure enabling systematic study of cellular behavior [24, 25, 26].

Inverted-colloidal-crystals are formed by infiltrating a hydrogel into the colloidal crystal template and removing the template by solvent extraction [27]. A common choice for the hydrogel is polyacrylamide gel because of its simplicity, optical quality, and ideal mechanical properties [28]. Additionally, the stiffness of a polyacrylamide gel can be modified to mimic the
rigidity of different tissues. For example, altering the concentration of crosslinkers can change the elastic modulus from 2.2 kPa to 4.4 kPa to represent the environment of premalignant breast and breast tumor respectively [29]. To match the surface chemistry of the scaffolds with that of native tissue, the scaffolds were coated with protein. We chose to use type I collagen, reasoning that the connective tissue is highly enriched with this protein [30]. The cells studied were from the breast cancer cell MDA-MB-231 line because of their high permeative and invasive capabilities [18].

In many instances of metastasis, cancer cells encounter different physical environments as they detach from their primary tissue and migrate to a distant site [31]. Examples of these different physical environments, or “spatial gradients,” include tissue organization and branching of vessels in the vasculature. In particular is the migration through differing pore sizes in the extracellular matrix. Interestingly, metastatic cells have been found to have a higher probability of permeation into small regions than non-metastatic cells [18]. To understand how these cells are able to navigate tissue so efficiently, this thesis aims to characterize the mechanical responsiveness of cancer cells when encountering spatial gradients, particularly in the context of squeezing through different pore sizes. Cellular response to physical gradients will be quantified by measuring the tractions of these cells. It is hoped that these measurements will provide insight into how cancer cells move through a pore that is smaller than its cross section. If cell tractions do not increase as these cells squeeze through smaller pores, perhaps the cells harbor the ability to reorganize their cytoskeleton and remodel their shape until they can pass through [4]. If traction forces do increase, then further studies should be done to determine the mechanism in which cancer cells can generate increased traction forces.
Chapter 2: Materials and Methods

2.1 Fabrication of Three-Dimensional Hydrogel Scaffold

Scaffolds with inverted-colloidal-crystal geometry were prepared by infiltrating a hydrogel into the interstices of glass microspheres. Pores were formed by dissolving the microspheres with a solvent that did not react with the hydrogel. Before infiltration of hydrogel, microspheres must first be partially fused. Once fused, etching of the beads will leave interconnecting channels that serve as migration pathways for the cells. Various bead sizes were used to create a changing environment for the cells. The sizes were chosen based on the average cell width (Figure 2) so that the cells could encounter three types of environment: one in which the pore sizes are smaller than the cells, one in which the pores are bigger than the cells, and one in which the pores are just the “right” size. The cell widths were measured to be around 10 µm and the bead sizes were chosen accordingly: 3-6 µm, 10 µm, and 35 µm (Table 1).

Figure 2. Image of a MDA-MB-231 cell. Confocal image of a single carcinoma cell taken with A1 Nikon confocal microscope at 40X magnification. The image shows that when adhered to a surface, the cell is approximately 70 µm in length and 10 µm in width. These dimensions were taken into account when choosing pore sizes.
Table 1. **Mean and standard deviation of soda lime glass beads.** Soda lime glass beads were obtained from Prizmalite (New York, NY). These beads came in three different sizes labeled 3-6 µm, 10 µm, and 35 µm. Thirty beads (n = 30) from each group were measured using a confocal microscope. The means and standard deviations are reported below.

<table>
<thead>
<tr>
<th>Manufacturer’s size</th>
<th>Mean (µm)</th>
<th>Standard deviation (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-6 µm</td>
<td>4.70</td>
<td>1.06</td>
</tr>
<tr>
<td>10 µm</td>
<td>6.20</td>
<td>1.59</td>
</tr>
<tr>
<td>35 µm</td>
<td>22.05</td>
<td>3.77</td>
</tr>
</tbody>
</table>

Glass beads (Prizmalite, New York, NY) were dropped into a glass beaker (height: 33 mm; OD: 25 mm) until the thickness reached approximately 1 millimeter. A height well below 1 millimeter created scaffolds that were later hard to remove from the beaker, and a height well above 1 millimeter led to scaffolds with beads that were only partially dissolved. The beads were annealed at 700°C for 1.5 hours to partially melt the spheres and fuse them together.

The hydrogel must provide an environment as close as possible to the environment in which the cells are found. This means that the hydrogel must have a pH close to 7.4, have an elastic modulus that matches the tissue in which the cells are found, and display similar surface chemistry as the tissue. Since the elastic modulus for breast tumor is ~4.1 kPa, an initial hydrogel of 5 kPa was chosen [29]. A concentration of 8% (w/v) acrylamide (Bio-Rad) and 0.1% N,N-methylene-bis-acrylamide (BIS) was mixed with Irgacure 2959 (Ciba), a photoactivated crosslinker, according to the ratios shown in Table 2. In addition, 0.2 µm green fluorescent FluoSpheres (Invitrogen) were added to the solution for the visualization of scaffolds under fluorescence microscopy. The 5 kPa hydrogel however, was too soft to be handled so the elastic modulus was increased to 10 kPa for easier handling (Table 2).
Table 2. Mixing ratios of 1000 µL polyacrylamide gel precursor for an elastic modulus of 5 kPa and 10 kPa.

<table>
<thead>
<tr>
<th></th>
<th>5 kPa</th>
<th>10 kPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% Acrylamide</td>
<td>125 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>2% BIS</td>
<td>50 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>Deionized water</td>
<td>765 µL</td>
<td>690 µL</td>
</tr>
<tr>
<td>FluoSpheres</td>
<td>60 µL</td>
<td>60 µL</td>
</tr>
<tr>
<td>Irgacure</td>
<td>5 mg</td>
<td>5 mg</td>
</tr>
</tbody>
</table>

The colloidal templates were infiltrated with 300 µL of gel precursor and polymerized via UV-light irradiation for 10 minutes. After polymerization, the hydrogel piece containing microspheres was removed from the beaker with a spatula and submersed in 7% hydrofluoric acid for 12 hours. After 12 hours, the hydrofluoric acid completely dissolved the glass beads while leaving the hydrogel scaffold intact. To adjust the pH of the scaffold to physiological pH (pH 7.4), the scaffolds were first rinsed with deionized water and then immersed in 7% sodium hydroxide for at least 12 hours. Sodium hydroxide was changed every hour for 5 hours to prevent the saturation of acid. The scaffolds were then left in 1X phosphate-buffered saline until later use.

To conjugate proteins to scaffolds, scaffolds must first be coated with 1 mg/ml sulfo-SANPAH, a heterobifunctional crosslinker. Upon irradiation with UV-light for 15 minutes on both sides of the hydrogel piece, the sulfo-SANPAH is attached to the scaffold in the mechanism described in Figure 3. When the nitrophenyl azide group is exposed to UV light, it forms a nitrene group which easily inserts into the carbon-hydrogen covalent bond of polyacrylamide [32]. The sulfosuccinimidyl group at the other end of sulfo-SANPAH reacts with primary amines of collagen (Figure 4).
Figure 3. Reaction of sulfo-SANPAH with polyacrylamide upon photoactivation. When the nitrophenyl azide group (circled in red) of sulfo-SANPAH is exposed to UV light, it forms a nitrene group that reacts with polyacrylamide. The figure was created in ChemDraw Ultra 12.0.
Figure 4. Reaction of sulfo-SANPAH with collagen. The sulfosuccinimidyl group (circled in blue) at one end of sulfo-SANPAH reacts with primary amine groups of collagen to form a stable amide bond. The figure was created in ChemDraw Ultra 12.0.
2.2 MDA-MB-231 Cell Culture

Cells of the MDA-MB-231 line were suspended in Dulbecco’s Modified Eagle Medium (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen) and 0.1% solution containing penicillin and streptomycin (Invitrogen). Cells were passaged, or transferred from one culture flask to another, once they reached high confluence (Figure 5).

![Figure 5. Images of MDA-MB-231 culture before and after cell passaging.](image)

(a) Low Density MDA-MB-231 Culture  
(b) High Density MDA-MB-231 Culture

Before passaging, 50 mL of culture medium and 5 mL of trypsin (Sigma) was warmed in a 37°C water bath for 30 minutes. The cell culture flask was then removed from the incubator and the culture medium was removed. 5 mL of trypsin was pipetted into the flask and the flask was returned to the incubator for 5 minutes. After 5 minutes, the flask was observed under a microscope to ensure that the cells have detached from the flask. If the cells were detached, 10 mL of the warm culture medium was pipetted into the flask to halt the trypsinization reaction.
The resulting solution from the flask was transferred to a conical tube and centrifuged at 2000 rpm for five minutes. After centrifugation, a cell pellet sat on the bottom of the tube. The supernatant above the pellet was aspirated and the pellet was re-suspended in 7 mL of warm culture medium. 1 mL of the resulting mixture was transferred into a new flask with 15 mL of medium in it. The flask was then returned to the incubator.

2.2.1 Labeling Cells with Fluorescent Dye

DiI is a fluorescent dye used to label plasma membrane so that cells can be detected under fluorescence microscopy. Too much of the dye can be toxic to cells so only a small amount of dye (10 µL dye for every 1 mL solution) was used. Once the dye has been added to the medium, the resulting solution was incubated at 37°C for 10 minutes. The solution was then centrifuged for 5 minutes and the solution above the cell pellet was removed. 10 mL of medium was added above the cell pellet and mixed before the step above was repeated again. A repeat of the previous step is important to ensure removal of excess dye that could be toxic to the cells.

2.3 Fabrication of Two-Dimensional Hydrogel Scaffold

Two-dimensional hydrogel scaffolds were also fabricated so that cell tractions exerted on planar substrates could be compared to those exerted on 3D hydrogel scaffolds. Polyacrylamide gel was first pipetted onto a clean microscope slide. A coverslip chemically treated for gel attachment was then laid on top of the gel causing the gel to spread uniformly between the slide and the coverslip. After the gel had polymerized, the sandwiched coverslips was immersed in deionized water for easy removal of the bottom slide. The mixing ratio for this gel precursor was
slightly different from that for the three-dimensional scaffold. Instead of using the photoactivated
crosslinker Irgacure, a mixture of ammonium persulfate (APS) and tetramethylethylenediamine
(TEMED) was used to catalyze the polymerization of acrylamide solutions into gel matrices.

Table 3. Mixing ratios of polyacrylamide gel precursor for an elastic modulus of 10 kPa.

<table>
<thead>
<tr>
<th>40% Acrylamide</th>
<th>2% BIS</th>
<th>Deionized water</th>
<th>FluoSpheres</th>
<th>APS</th>
<th>TEMED</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 µL</td>
<td>50 µL</td>
<td>690 µL</td>
<td>60 µL</td>
<td>12.5 µL</td>
<td>5 µL</td>
</tr>
</tbody>
</table>

The scaffolds were then coated with sulfo-SANPAH and collagen in the same procedure
described earlier. The general approach for this scaffold design is summarized in the following
figure:

**Figure 6. Summary of procedures for fabricating 2D hydrogel scaffold.** (a) A glass
microscope slide was chemically activated to produce a (b) surface that allowed gel attachment.
(c) Polyacrylamide gel was pipetted onto the slide and (d) a hydrophobic coverslip was placed on
top of it to create a uniform layer of gel.
2.3.1 Chemical Activation of Coverslips

Coverslips were treated chemically to allow covalent attachment of the gel to the coverslip. Coverslips were first rinsed with ethanol, then placed in a Petri dish containing 0.5% (v/v) 3-aminopropyltrimethoxysilane (Gelest) in ethanol for five minutes. The coverslips were then removed with a tweezer and rinsed again with ethanol before immediately submersed in a solution of 0.5% (v/v) glutaraldehyde (Polysciences, Inc) in water for 30 minutes. After 30 minutes, the coverslips were removed, rinsed with deionized water, and left to dry for several hours at 60°C. Microscope slides were also treated chemically but instead of promoting gel adhesion, slides were made hydrophobic for easy gel removal after sandwiching. Slides were submerged in a Petri dish containing 0.5% (v/v) acetic acid in hexane and 2.5% (w/v) (tridecafluoro-1, 1, 2, 3-tetrahydrooctyl)-triethoxysilane (Gelest). After one minute in solution, the slides were removed and left to dry at room temperature.

2.4 Cell Imaging

All traction force visualizations were done using a Nikon AR-1 confocal system with a 40X CFI planar fluorite air objective. Imaging was done with two laser lines: a green Helium Neon (543 nm) laser for imaging microparticles inside the scaffold and a red Helium Neon (633 nm) for imaging the fluorescently labeled cells. Physiological conditions were maintained during imaging. A feedback controlled heater maintained the temperature at 37°C and addition of arterial blood gas (5% CO₂, 20% O₂, 75% N₂) maintained cell media pH and CO₂ levels.
2.5 Three-dimensional Traction Force Microscopy

Determination of cell tractions involved several steps (Figure 7). First, the location of fluorescent particles embedded in the polyacrylamide gel were tracked and recorded using laser scanning confocal microscopy. Confocal microscopy is a powerful imaging technique that allows the construction of 3D images due to its increased contrast and resolution compared to that of conventional microscopy. Such resolution is possible because a confocal microscope only collects light from the focal plane, blurring out-of-plane light. Three-dimensional image stacks were acquired every 10 minutes for 7 hours. Displacements from these image stacks were determined with digital volume correlation, a technique that compares two volume images of a substrate obtained before and after mechanical loading. A more detailed discussion on the technique can be found elsewhere [7]. Once the displacement field data were obtained, the strain and stress tensors were calculated as described in the results section. Finally, cell-traction forces were calculated by matrix multiplication of the normal vector with the stress tensor.

Figure 7. Overview of 3D TFM. A flow chart illustrating the procedures used in 3D TFM.
Chapter 3: Results and Discussion

3.1 Scaffold Results

Inverted colloidal crystal hydrogels were synthesized by infiltrating polyacrylamide gel into annealed glass beads and etching the beads to leave spherical cavities. Annealing was done at different temperatures and time intervals to obtain maximal results. Results obtained from different temperatures and time intervals are documented in Table 4. An initial temperature of 800°C was chosen as it was believed to easily melt the glass beads since the softening temperature of the beads is 700°C.

Table 4. Annealing of beads done at different temperatures and time intervals. Soda lime glass beads were annealed at six different conditions. Results for each condition are tabulated and compared to determine the optimal temperature and time interval for annealing. The order in the table follows the order in which the conditions were tried.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (hours)</th>
<th>Observations</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>800</td>
<td>2</td>
<td>Beads melted and stuck to the bottom of the beakers.</td>
<td>×</td>
</tr>
<tr>
<td>800</td>
<td>1</td>
<td>Beads were stuck to the beakers and could not be removed.</td>
<td>×</td>
</tr>
<tr>
<td>750</td>
<td>1.5</td>
<td>Beads fused together to form a crystal template that could be removed from the beaker but scaffolds produced from these templates had “cracks” instead of pores.</td>
<td>×</td>
</tr>
<tr>
<td>750</td>
<td>1</td>
<td>Beads fused together to form a crystal template that could be removed from the beaker but scaffolds produced from these templates had “cracks” instead of pores.</td>
<td>×</td>
</tr>
<tr>
<td>700</td>
<td>1</td>
<td>Beads did not fuse together.</td>
<td>×</td>
</tr>
<tr>
<td>700</td>
<td>1.5</td>
<td>Surfaces of beads melted and stuck to the surfaces of adjacent beads.</td>
<td>✓</td>
</tr>
</tbody>
</table>
A final temperature of 700°C and a time of 1.5 hours were chosen as beads annealed at temperatures less than 700°C did not fuse together and beads annealed at temperatures greater than 700°C produced cracks. A fusing of beads was necessary to create a crystal template that when etched, left connected pores for cells to migrate through (Figure 8).

Once the scaffolds have been prepared, cells from the MDA-MB-231 line were seeded on the scaffolds and left in an incubator at 37°C overnight. The scaffolds, along with cellular growth, were observed for a duration of up to two weeks. Confocal images of cell adherence indicated that cells remained viable in the scaffolds (Figure 9). Adherence was determined by tapping the petri dishes; cells that remained stationary during tapping were considered “attached” to the scaffolds and alive while those that moved and floated in solution during tapping were considered dead. These images confirm that the scaffolds produce a physiological environment for the cell and hence, are viable models for studying the role of the microenvironment on cell migration.
Figure 9. (a) Image of hydrogel scaffold in the XY plane under a confocal microscope at 10X magnification. (b) Three-dimensional view of a scaffold. The green represents the hydrogel which was labeled with 0.2 µm green fluorescent Fluospheres and the red dots are the cells which were labeled with DiI. Cells can be found to grow as deep as ~180 µm.

Unfortunately, most of the scaffolds had pores greater than 50 µm, and even 100 µm after etching. The large pore sizes most likely resulted from the long etching time. These pore sizes were too big compared to the width of carcinoma cells. Because of the large pore sizes, most cells fell right through the pores upon seeding and were not able to adhere onto the scaffold. Even if some cells did adhere, as shown in Figure 9, the pore sizes were still too large for the hypothesis to be tested. Due to time constraints, traction measurements were done on 2D polyacrylamide substrates instead. Traction measurements on these planar substrates established a baseline estimate of traction forces on 3D substrates.
3. 2 Calculation of Cell Traction

In the simplest case, the traction force a cell exerts on a substrate can be modeled with Hooke’s law:

\[ F = -kx \]  

(1)

where \( k \) is the spring constant and \( x \) is the displacement of the spring (Figure 10A). The substrate is assumed to be isotropic, meaning its properties are the same in all directions. In this model, the cell displaces the matrix surface by a distance \( x \) to produce a force \( F \) depending on the “stiffness” \( k \) of the material. If the matrix is relatively soft (small \( k \)), the cell can easily displace the substrate with little force. On the other hand, if the matrix is relatively stiff (large \( k \)), a greater force is required to displace the substrate. Instead of “one” spring, the scaffold can be modeled as an array of springs where the number of springs in parallel is proportional to the cross sectional area \( A \) of the substrate (Figure 10B).

**Figure 10. Modeling cell tractions with Hooke’s law.** (A) An isotropic material can be modeled as a spring with spring constant \( k \). When the cell pulls on the substrate with a force \( F_p \), it displaces the surface by a distance \( x \) to produce an equal and opposite force \( F_t \) on the substrate. (B) A scaffold can be modeled as an array of springs so that Hooke’s law, \( F = -kx \), can be rewritten as: \( \frac{F}{A} = E \frac{\Delta L}{L} \) where \( E \) is proportional to the spring constant \( k \). This image was modified from Georges and Janmey [33].
Hence, the force applied to each spring is the total applied force divided by the cross sectional area. Hooke’s law can then be written as:

\[
\frac{F}{A} = E \frac{\Delta L}{L}
\]  

(2)

where the displacement \(\Delta L\) has been normalized by the initial length of the material and \(E\) is defined as the elastic modulus of a material. Equation 1 is more commonly known as:

\[
\sigma = E \varepsilon
\]  

(3)

where stress \((\sigma)\) is \(F/A\), strain \((\varepsilon)\) is \(\Delta L/L\), and \(E\) is the Young’s modulus of a material. The stress can be understood as the average force per unit area. The strain is the relative change in shape or length of an object due to externally-applied forces. When a object is stretched, the strain is positive and when the object is compressed, the strain is negative.

This simple model however, is limited to one dimension. In three dimensions, we need to take into account interactions in all directions. For example, when a substrate is pulled in the z-direction, there tends to be a contraction in the x- and y- direction. This phenomenon, called the Poisson's effect, is caused by movements between molecules to accommodate stress. Thus, when a material elongates in the direction of load, it tends to shorten in the other directions. The ratio of contraction (in the direction perpendicular to the applied load) to the extension (in the direction of the applied load) is known as Poisson’s ratio \((\nu)\). Hence, if the applied load is in the z-direction, the strains in the x- and y- direction are:

\[
\varepsilon_x = \varepsilon_y = -\nu \varepsilon_z
\]  

(4)

The stress tensor is related to the strain in the following relationship:

\[
\sigma_{ij} = C_{ijkl} \varepsilon_{kl}
\]  

(5)
where $C_{ijkl}$ is the elasticity or stiffness tensor. For an isotropic, homogeneous and linear elastic material, the stress tensor is [32]:

$$
\sigma_{ij} = \frac{E}{1+v} \left( \varepsilon_{ij} + \frac{\nu}{1-2\nu} \varepsilon_{kk} \delta_{ij} \right)
$$

(6)

where $E$ is the elastic modulus of the material, $\nu$ is 0.45 for a typical polyacrylamide gel, and $\delta$ is the Kronecker delta:

$$
\delta_{ij} = \begin{cases} 
0, & \text{if } i \neq j \\
1, & \text{if } i = j 
\end{cases}
$$

(7)

i and j refers to the axis and can take on the values 1-3 (Figure 11).

**Figure 11. Stress directions in 3D.** When measuring cell tractions in 3D, interactions in all dimensions need to be taken into account. The numbers 1, 2, and 3 represent the X, Y, and Z axis respectively. Each stress value is denoted with two numbers: the first number represents the X, Y, or Z plane while the second number represents the strain direction on that specific plane.

Once the stress tensor has been found, the traction force vector can be calculated by multiplying the stress tensor by the normal vector of a particular surface plane along which the tractions act,

$$
T = \sigma \cdot n
$$

(8)

Finally, the magnitude of the total traction force can be calculated as:

$$
T = \sqrt{(T_1)^2 + (T_2)^2 + (T_3)^2}
$$

(9)
This magnitude has two components: the in-plane traction force vector and the out-of-plane traction force vector. The in-plane traction forces are forces exerted on the same plane as the substrate while the out-of-plane traction forces are forces exerted on the plane normal to the substrate. The in-plane and out-of-plane traction force magnitudes are:

\[ T_{\text{in-plane}} = \sqrt{(T_1)^2 + (T_2)^2} \quad (10) \]

\[ T_{\text{out-of-plane}} = \sqrt{(T_3)^2} \quad (11) \]
3.3 Cell Traction Data on 2D Control Samples

For reasons mentioned earlier, only tractions on a 2D substrate were measured. However, it is expected that the data obtained will share similar trends as tractions on 3D hydrogel scaffolds. MDA-MB-231 breast cancer cells were seeded onto a 2D polyacrylamide gel with a Young’s modulus of ~5 kPa. The Young’s modulus was chosen to mimic the rigidity of breast tumor tissue. Displacement measurements were computed using the LSCM-DVC technique as discussed in the “Methods” section. Each cell was visualized simultaneously with the displacement field. The image below shows the single motile cell tracked during the experiment along with the detected displacement fields in all three planes (Figure 12). There are several features to note: the longer dimension of the cell is approximately 80 µm, the direction of the cell locomotion is from the left to the right, and the color contour plots represent the magnitude of the displacement vector. A peak displacement magnitude, represented in red, can be found at the leading edge of the cell.

Figure 12. Displacement contours of a single motile MDA-MB-231 cell. On the left is a confocal image of the MDA-MB-231 cell recorded during time-lapse imaging experiment. On the right are the displacement contours resulting from the motile cell shown on the left. The vectors represent the in-plane displacement vectors and the color bar represents the magnitude of the displacements in three dimensions.
Once the field displacements were determined as shown in Figure 12, the stress and strain tensors were calculated using equation 6. From the stress tensors, the magnitudes of the total traction force vectors were obtained and plotted in Figure 13.

**Figure 13. Traction contours of migrating MDA-MB-231 cells.** (a)-(d) show traction force contours during cell migration for four time intervals. The cell, outlined in white, is superimposed on the traction force field data. Magnitudes of the traction force vectors are represented in the color bars in pN/μm².
Figure 13 shows the magnitude of the total traction force vector for four time intervals: \( t_1 = 15 \) minutes, \( t_2 = 75 \) minutes, \( t_3 = 225 \) minutes, and \( t_4 = 315 \) minutes. These intervals were chosen to represent contractile forces exerted by the cell during the initial, intermediate, and final time measurements. In other words, we wanted to see how the traction forces changed as time progressed. The specific time points were also chosen to capture the morphological change of the cell as it migrated. The magnitude of the total traction force vector in all time frames have a peak value of \( \sim 350 \) pN/\( \mu \text{m}^2 \); this peak magnitude decays dramatically away from the cell edge.

The data also show localized cell traction directly underneath the cell’s leading and trailing ends at all time intervals. This localization trend is in agreement with previously published two- and three-dimensional studies [34,35]. Localization of cell tractions most likely correspond to local force transmission at focal adhesions. Large tractions at all time points indicate that the cell is constantly exerting force, probably to deform the substrate for easier migration. This hypothesis is likely as Harris et al. found that cells exert much stronger forces than needed for locomotion [36,37]. It is interesting to note that peak magnitudes of tractions are only found at either the leading or trailing end of the cell and not simultaneously at both ends. This suggests that the cell exerts force, or “pushes,” with one end to establish new contact with the substrate and releases force to break old contact at the other end.

Although the data presented here already highlights the dynamic interaction between a cell and its environment, a planar substrate is not sufficient in understanding a cell’s migration machinery in native tissue. An environment allowing cell migration in the depth direction is needed. One such environment is a 3D hydrogel scaffold presented in the “Methods” section. Not only will the scaffolds provide information on migration in the depth direction, but it will
also provide insight on how a cancer cell “squeezes” through small pores. If cancer cells have the ability to reshape its cytoskeleton and change shape to “fit” through the pores, this fact will be illustrated by similar magnitudes in cell traction. On the other hand, greater cell tractions through small pores indicate the cells’ ability to remodel the matrix during invasion.
Chapter 4: Future Work

4.1 Immunohistochemistry

An immunohistochemistry procedure should be done to confirm a uniform distribution of collagen I on the scaffolds. If such distribution does not exist, then we need to consider the influence of chemical gradients on cell tractions. Immunohistochemistry is a process of detecting antigens (proteins) based on the binding of an antibody to an antigen. The antibody is usually tagged with a fluorescent compound so that once the antigen-antibody binding occurs, it is demonstrated with a colored histochemical reaction visible by fluorescence microscopy. The procedure carried out would be an indirect one where an unlabeled primary antibody first binds to the target antigen, then a secondary antibody reacts with the primary antibody (Figure 14).

![Figure 14. Indirect method of immunohistochemistry](image)

**Figure 14. Indirect method of immunohistochemistry.** The indirect method involves using a primary antibody to react with the antigen (collagen). A secondary antibody is then used to react with the primary antibody. The histochemical reaction can be viewed with microscopy through the fluorescent tag on the secondary antibody.

4.2 Scaffold Improvement

Cell tractions for 3D hydrogel scaffolds were not measured because final scaffolds had pores much larger than the ones desired. Most pores fell between the range of 50 to 100 µm
(Figure 8), which were too large for the cells to attach to and adhere upon seeding. Scaffolds after etching also became rough and non-uniformities were observed at the bottom of the etched cavities (Figure 15).

It is hypothesized that the composition of the soda lime glass used is what caused the defects. Soda lime glass contains CaO, MgO, and Al₂O₃, all of which form insoluble products in hydrofluoric acid (HF):

\[
\begin{align*}
    \text{CaO} + 2 \text{HF} & \rightarrow \text{CaF}_2 + \text{H}_2\text{O} \\
    \text{MgO} + 2 \text{HF} & \rightarrow \text{MgF}_2 + \text{H}_2\text{O} \\
    \text{Al}_2\text{O}_3 + 6 \text{HF} & \rightarrow 2\text{AlF}_3 + 3\text{H}_2\text{O}
\end{align*}
\]

where CaF₂, MgF₂, and AlF₃ are all insoluble. These insoluble products may deposit onto the scaffold surfaces and create “rough” surfaces. A proposed solution is to add hydrochloric acid (HCl), which can transform the insoluble products into soluble ones:
CaF₂ + 2 HCl → CaCl₂ + 2 HF
MgF₂ + 2 HCl → MgCl₂ + 2 HF
AlF₃ + 3 HCl → AlCl₃ + 3 HF

It has been shown that optimal etching quality for soda lime glass is achieved in a 10:1 HF to HCl solution [38]. If etching conditions can be improved, then scaffolds may be left in the etching solvent for a shorter time period. Perhaps a shorter time interval in hydrofluoric acid will improve the “smoothness” of scaffolds and produce scaffolds with smaller pore sizes.

4.3 Cell Tractions for 3D Hydrogel Scaffolds

Once 3D hydrogel scaffolds with the desired pore sizes have been synthesized, cancer cells seeded onto these scaffolds will be measured for their traction forces. The migratory behavior of cells on 3D substrates will be compared to that on the 2D substrates discussed above. Further experiments would need to be conducted to determine a method for attaching scaffolds onto glass sides during imaging. Attachment of scaffolds to a slide is important when acquiring image-stacks so that any movement can be solely attributed to cell migration.
References


